Ionic Regulation of Critical Cellular Processes in Non-Excitable Cells

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IONIC REGULATION OF CRITICAL CELLULAR PROCESSES IN NON-EXCITABLE CELLS

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the College of Arts and Sciences at the University of Kentucky

By
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ABSTRACT OF DISSERTATION

IONIC REGULATION OF CRITICAL CELLULAR PROCESSES IN NON-EXCITABLE CELLS

There are long-standing hypotheses that endogenous ion currents act to control cell dynamics in development, wound healing and regeneration. However, the mechanisms employed by cells to detect the electric field (EF) and translate it into a discernable message to drive specific cell behaviors, such as migration, proliferation and differentiation, are not well understood. A better understanding of how cells are able to sense EFs and react to them is vital to understanding physiological mechanisms are involved in regeneration. Ion channel signaling provides a reasonable suspect for mediating these effects based on their documented involvement in proliferation, migration and differentiation.

To investigate mechanisms underlying ionic regulation of critical cellular processes in non-excitable cells, a novel, in vivo assay was developed to screen multiple pharmacological inhibitors of ion channels during larval A. mexicanum tail regeneration. This assay was used to identify individual channels that were then targeted for further analysis regarding their involvement in the regenerative process. Chapter 2 presents data from a study that indicates that a wound-like response can be generated in an invertebrate model by application of exogenous, low-amplitude sine-wave electrical stimulation. This was characterized by recruitment of hemocytes at the stimulation site which was dependent on voltage-gated potassium channels. Chapter 3 presents data from a comprehensive and systematic screen of pharmacological compounds against larval salamander tail regeneration that indicates 8 specific target ion channels. This chapter also describes results indicating specific mechanisms by which these channels may be perturbing regeneration. Chapter 4 presents data that indicate that the Anoctamin 1 channel identified in the aforementioned screen is a regulator of cellular proliferation. This is shown to be accomplished via amplification of intracellular calcium surges and a subsequent increase in the activity of the p44/42 MAPK signaling cascade.
KEYWORDS: Proliferation, Bioelectricity, Ion Channels, MAPK, Regeneration, Wound-Healing, and Macrophages
IONIC REGULATION OF CRITICAL CELLULAR PROCESSES DURING WOUND-HEALING, REGENERATION AND BEYOND

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4-19-2017
Date
For my wife and children:
Heidi Rae, Sawyer Michael and Ada Morgan
“Whenever you find yourself on the side of the majority, it is time to pause and reflect.”

— Mark Twain

“It may be that when we no longer know which way to go that we have come to our real journey. The mind that is not baffled is not employed. The impeded stream is the one that sings.”

— Wendell Berry

“Yesterday’s weirdness is tomorrow’s reason why.”

— Hunter S. Thompson
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CHAPTER 1
ION CHANNEL REGULATION OF CRITICAL CELLULAR DYNAMICS:
IMPLICATIONS ON WOUND HEALING AND REGENERATION

Brandon Michael Franklin

**Abbreviations used:** EF (electric field), TEP (trans-epithelial potential), $V_{\text{mem}}$ (plasma membrane potential), $\text{Na}^+$ (sodium cation), $\text{K}^+$ (potassium cation), DC (direct current), $\text{Cl}^-$ (chloride anion), ENaC (epithelial sodium channel), CFRT (cystic fibrosis transmembrane receptor), $\mu$A (micro ampere), cm$^2$ (centimeter squared), mV (millivolt), mm (millimeter), PI(3)K$\gamma$ (phosphatidylinositol-4,5-bisphosphate 3-kinase gamma), PTEN (phosphatase and tensin homolog), AC (alternating current), IGF-II (insulin like growth factor 2), VEGF (vascular endothelial growth factor), ECM (extra cellular matrix), ES (electrical stimulation), $\text{Ca}^{2+}$ (calcium cation), CAMKII (calmodulin-dependent protein kinase II), SOCE (Store-operated calcium entry), IP$_3$ (inositol 1,4,5-trisphosphate), IP$_3$R (IP$_3$ receptors), ER (endoplasmic reticulum), STIM1 (stromal interaction molecule 1), CRAC (calcium release activated channels), $\text{Kv}$ (voltage-gated potassium channel), $\text{H}_2\text{O}_2$ (hydrogen peroxide), DIDS (4,4′-Diisothiocyanatostilbene-2,2′-disulfonic acid), Ano1/2 (anoctamin 1 or 2 protein), MAPK (mitogen activated protein kinase), EGFR (epidermal growth factor receptors), CAMK (calmodulin dependent protein kinase), $\text{Kca}$ (calcium activated potassium channel), $\text{Nav}$ (voltage-gated sodium channel), PCR (polymerase chain reaction), HEK293 (human embryonic kidney cells line 293), Erk (extra cellular signal regulated kinase)

**Keywords:** Ions, Ion channels, Electric fields, Wound-healing, Regeneration
1.1 Introduction

Urodele amphibians have a unique ability to activate multipotent resident stem cell populations and/or to dedifferentiate somatic cells in order to regenerate a perfect replacement for a lost limb or tail (Muneoka, Fox et al. 1986, Echeverri, Clarke et al. 2001, Morrison, Lööf et al. 2006, Morrison, Borg et al. 2010, Sandoval-Guzmán, Wang et al. 2014). While salamanders are the champion regenerators of vertebrate lineages, there are many species that employ a diverse array of mechanisms and cell sources to replace lost tissues after injury (Sanchez Alvarado 2000, King and Newmark 2012). Despite this diversity, a common theme rings through; cellular proliferation and migration are critical process in all models of regeneration. How cells interpret signals (and what these signals are) as guidance cues for proliferation, migration and cell fate decisions remain unclear. A better understanding of major regulatory mechanisms for these critical cellular behaviors is an essential step that may lead to recapitulation of these phenomenon in mammalian models, and ultimately to the development of clinically relevant applications.

There are long-standing hypotheses that endogenous ion currents act to control cell dynamics in development, wound healing, and regeneration (Jaffe and Nuccitelli 1977, Borgens, Vanable et al. 1979, Jaffe 1981, Özkucur, Epperlein et al. 2010). However, the mechanisms employed by cells to detect the electric
field (EF) and translate it into a discernable message to drive specific cell behaviors are not well understood. Ion channel signaling provides a reasonable target for mediating these effects based on the close relationship between ion channels, endogenous EFs, and membrane potentials. Also, documented involvement of ion channels in proliferation, migration, and differentiation is relevant to this discussion (Wondergem, Gong et al. 2001, Wang, Wang et al. 2002, Tao, Lau et al. 2008, Mao, Chen et al. 2009, Wei, Akerman et al. 2011).
1.2 Electric Fields during Regeneration and Wound Healing

Wound-induced EFs were first observed by the German physician and physiologist, Emil Du-Bois Reymond, in 1843 (du Bois-Reymond 1843). The act of wounding instantaneously creates endogenous EFs by disrupting trans-epithelial potentials with current flowing out of the wound area as seen in Figure 1.1B (McCaig, Rajnicek et al. 2005, Zhao 2009, Wang and Zhao 2010, Messerli and Graham 2011, Sun, Reid et al. 2011). Wound healing and regeneration are dependent on intrinsic wound-induced EFs in a variety of animals and tissues, including: rat cornea (Reid, Song et al. 2005), *xenopus* tail regeneration (Adams, Masi et al. 2007, Tseng, Beane et al. 2010), newt limb regeneration (Jenkins, Duerstock et al. 1996) and wound healing (Chiang, Cragoe et al. 1991). The removal of this EF through reversal of ion current via exogenous electrical stimulation (Jenkins, Duerstock et al. 1996) or pharmacological inhibition of ion channels (Adams, Masi et al. 2007, Tseng, Beane et al. 2010) abolishes regenerative capacity and is sufficient to induce regeneration in the non-regenerative refractory period of *xenopus* development. Additionally, regenerative outgrowth in the mammalian limb has been shown to be significantly enhanced, albeit abnormal, by exogenous application of direct current EF (Stephen D 1981). Despite this large amount of evidence indicating a role of endogenous EFs in regeneration and wound healing little is known regarding the mechanisms driving its influence.
Regeneration is a complex process involving reconstruction of several different tissue types from resident and dedifferentiated stem cells. Effective direction of cell migration and proliferation in a wound is vital for healing and regeneration. Several mechanisms are proposed as responsible for directing these cellular behaviors: injury stimulation, gradients of chemoattractants, contact inhibition release, wound void, growth of adjacent cells pushing cells into a wound, and changes in mechanical force following injury (Zhao 2009). It is likely that healing and regeneration is the product of these forces acting together and EF may play a significant role considering its ability to override other directional cues (Zhao, Song et al. 2006). There is a wealth of literature that indicates a role for EFs in guiding cell migration (McCaig and Zhao 1997, Mycielska and Djamgoz 2004, McCaig, Rajnicek et al. 2005). Galvanotaxis has been shown to be independent of cytokine receptors known to be involved with chemotaxis which leads to the conclusion that EF directed migration is not elicited in toto through modulation of cytokine gradients (Zhao, Jin et al. 2002). This suggests that the EF is able to communicate directly with the cell through membrane receptors. A better familiarity of how cells are able to sense EFs and migrate along them is vital to understanding the global physiology involved in bioelectric regulation of limb regeneration.
1.2.1 Generation of the Trans-Epithelial Potential (TEP) and the Wound Induced Electric Field

The plasma membrane potential ($V_{\text{mem}}$) is formed across cell membranes in nervous and muscle tissue by the activity of the Na$^+/K^+$ ATPase and the general impermeability of the plasma membrane to ion conductance. Each cycle of this pump results in the expulsion of three Na$^+$ ions from the cell and entry of two K$^+$ ions resulting in the net loss of one positive charge. This generates potential energy across the membrane and gives rise to the excitability of the cells of these tissues by creating a DC electric field upon modulation of the membranes permeability to Na$^+$ ions. These cells are then able to perpetuate this signal down the length of an axon resulting in nerve conduction in nervous tissue or instigate the opening of calcium channels leading to muscle contraction and the generation of force in muscle tissue.

The wound induced EF is generated via similar properties. The potential energy that gives rise to this EF is not stored across plasma membranes, but rather across sheets of epithelial cells held together by tight junctions which provide a high resistant barrier to ion conductance. Much of the work done to discover the molecular determinants of the TEP has been conducted in corneal epithelium, including: rat (Reid, Song et al. 2005, Zhao, Song et al. 2006), rabbit (Klyce 1975, Van der Heyden, Weekers et al. 1975) and frog (Zadunaisky 1966, Candia
and Askew 1968). Some common themes ring through these various models: (1) the primary ionic components of the TEP are Na⁺ and Cl⁻, (2) Na⁺ is actively transported from the apical to the basal surface of the epithelial sheet resulting in an inside positive potential and (3) the Na⁺/K⁺ ATPase is a critical component of this process. These findings are consistent with preliminary data from other models, including: *in vitro* reconstructed human skin (Moulin, Dubé et al. 2012) and amphibian limb regeneration (Borgens, Vanable et al. 1977).

This data taken together with basic knowledge of the Na⁺/K⁺ ATPase and other ion channels and transporters allows for the postulation of a working model of ion flow across epithelial sheets. Na⁺/K⁺ ATPase transporters are sequestered to the basal membrane of individual cells where they actively pump Na⁺ ions out of the cell. This is accompanied by passive flow of Na⁺ ions through epithelial Na⁺ channels (ENaC) situated exclusively on the apical side of the cell. The combined work of the Na⁺/K⁺ ATPase and ENaC channels results in a net movement of Na⁺ from the apical to the basal side of the cell. Also, Cl⁻ transporters work to actively transport Cl⁻ ions into the cell on the basal side of the cell while cystic fibrosis transmembrane receptor (CFTR) channels allow for passive efflux of Cl⁻ ions from the apical surface of the cell. Additionally, the cells are connected by tight junctions to prevent ion flow through the extracellular space. *(Figure 1.2)*
Establishment of the TEP by the aforementioned model of epithelial ion transport produces a potential to do work in the way of an electrochemical ion gradient (similar to those established across the membranes of excitable cells). Because of this any rapid alteration in the conductance of ions across the barrier will generate an ionic EF. This is accomplished in excitable cells via opening of voltage-sensitive or ligand-sensitive ion channels which allow for the free passive movement of specific ions across the plasma membrane driven by their reversal potential and the established electrochemical gradient. In the wound induced EF paradigm, the conductance is altered by the act of tissue insult that breaks the epithelial sheet allowing for free movement of all ions across the barrier driven by an electrical potential. The cells of the epithelial sheet surrounding the wounded area actively work to re-establish the TEP which works to “recharge the battery” and keep this EF going until the wounded epithelia has been closed. (Figure 1.1)

In most recorded cases of wound induced EFs, the EF is abolished upon closure of the wound with the exception of the regeneration competent red spotted newt. In this animal, which encloses the stump of an amputated limb in 5-10 hours, the peak EF leaving the stump was measured at 4 days following amputation. The mechanism driving this wound current is unknown but some hypotheses have been proposed. Either the wound epithelium is more electrically conductive and allows the charge generated by the surrounding mature skin to freely flow across is or there is a cohort of channels and pumps that are specifically expressed in
this wound epithelium region that drive the current. (Borgens, Vanable et al.
1977)
1.2.2 Recorded Wound Induced Electric Fields and the Consequence of Their Manipulation

The first recorded measurement of a wound induced EF was performed over 160 years ago by the German physician and physiologist and one of the fathers of modern electrophysiology Emil du Bois Reymond using a galvanometer of his own design. Acting as his own subject he created a small incision in his finger and using the contralateral finger as a reference he was able to measure a small current of approximately 1µA leaving the injured finger. (Figure 1.3) (du Bois-Reymond 1843, Finkelstein 2003) Several decades later, a former student of du Bois Reymond, Amedeo Herlitzka, reported on the experiments he performed following up his mentor’s work and was one of the first to conjecture a relationship between these wound induced currents and regeneration along with the renowned American physiologist Albert P. Mathews who measured electric currents in regenerating hydra. (Mathews 1903, Herlitzka 1910)

Following the work of Herlitzka in 1910, enthusiasm diminished for pursuing the bioelectric components of wound healing and regeneration which resulted in a dearth of literature on the matter that spans seven decades. This drought ended in 1977 when Richard Borgens, under the direction of Lionel F. Jaffe, found that driving a current out of freshly amputated forelimbs of normally non-regenerative adult frog species Rana pipiens and Xenopus laevis could lead to a partial
regenerate. This regenerated tissue was abnormal, but consisted of elongated bone as well as novel muscle, nerve, and connective tissues (cartilage and ligaments) (Borgens, Vanable et al. 1977, Borgens, Vanable et al. 1979). This group subsequently measured wound induced electric field in both the non-regenerative *R. pipiens* and the regeneration competent urodele amphibian, *Notophthalmus viridescens* (*red spotted newt*). Interestingly, they found that while currents generated by both species lasted approximately 10 day after amputation, the peak amplitude of the newt current (100 µA/cm²) was more than 2 times larger than that of *R. pipiens* (40 µA/cm²). (Borgens, Vanable et al. 1977, Borgens, Vanable et al. 1979) Additionally, reversal or reduction of the newt current by either pharmacological inhibition of ion channels or introduction of an exogenous electric field in the opposite direction reduces or inhibits regeneration. (Borgens, Vanable et al. 1977, Jenkins, Duerstock et al. 1996) This work establishes a notable relationship between the wound induced EF and regenerative capacity.

*Xenopus* tadpoles provide a unique model to study appendage regeneration. This is due to their distinctive ability to regenerate their tail during specific stages of development and the loss of this ability during other developmental stages. Specifically, they are not able to regenerate as stage 45 tadpoles but they are able to regenerate up until stage 45 and then after stage 47; up until they undergo metamorphosis and lose the ability for the remainder of their lifetime. The physiological differences in the cellular environment of the amputated tail in
the regenerating and refractory stage 45 tadpoles may lend insight into the molecular mechanisms that drive appendage regeneration. These animals also have a unique bioelectric profile as it relates to the wound induced EF. They produce the typical outward flowing current immediately following amputation with a peak amplitude of \(~4.5 \mu A/ cm^2\). In contrast to previously discussed models, this current is quickly dampened following amputation (2-4 hours) which correlates with formation of the wound epithelium. Interestingly, this current is reversed to an inward flowing current with a peak amplitude of about \(0.2 \mu A/ cm^2\) exclusively in regenerating, non-refractory animals. (Reid, Song et al. 2009)

This group also presented some mechanistic properties of the *Xenopus* wound current that diverged from previously discussed models. \(Na^+\) is required for the formation of this current, exhibited by a reduction in both wound current amplitude and regenerative capacity when tadpoles are placed in \(Na^+\) free bathing solutions but \(Cl^-\) ions are not required. This is contrasted by the requirement of both ions in previously discussed models with \(Cl^-\) being the dominant driver of the current. Although, the wound current’s requirement for \(Na^+\) ions is not mediated via “Na+ pumps” as it is in other models. (Reid, Song et al. 2009) These molecular differences driving the formation of the wound current could be responsible for the different physical characteristics it exhibits.

Wound induced EFs have been investigated in several other models of regeneration and wound healing. (Table 1.1)
1.2.3 Re-epithelialization of the Wound Site

Early on, investigations into the influence of bio-electric signaling on wound healing and regeneration was focused on two areas: (1) the channels and transporters responsible for creating the wound induced electric field, which has been discussed previously and (2) the ability of this endogenous field to influence re-epithelialization, which this section will focus on.

Galvanotaxis is a property exhibited by a number of cell types which permits them to migrate directly along an EF (Robinson 1985). Significant for the purposes of this study this group includes many epidermal and epithelial cells from a diverse array of species. The first of these models to be characterized were epithelial cells derived from *Xenopus* embryos, which when cultured in a direct current EF they migrated toward the cathode (Luther, Peng et al. 1983). Epidermal cells isolated from fish also migrate along an EF towards the cathode of a direct current EF. The elongation of the lamellipodia and ultimately the directional cell migration toward the cathode is dependent on functional Ca$^{2+}$ signaling but is not sensitive to alterations of extracellular K$^+$, suggesting that this characteristic is not influenced by the cells $V_{\text{mem}}$ (Cooper and Schliwa 1986). More recently, galvanotactic properties of cultured human keratinocytes has been characterized. These cells also migrate toward the cathode and require
functional Ca$^{2+}$ flux across the cell membrane (Fang, Farboud et al. 1998, Trollinger, Isseroff et al. 2002).

In all of these models, two characteristics stand out. (1) All of them operate in EFs of physiological magnitude between 10 and 100 mV/mm which correlates well with previously measured wound induced EFs (Table 1.1). (2) They migrate in the direction of the cathode, which in the aforementioned wound induced EF models would be toward the center of the wound (Figure 1.1B). In physiological context of wound healing and regeneration it may be more relevant to study how sheets of epithelial cells move in concert rather than the migratory properties of single cells (Zhao, Song et al. 2003). Importantly, collective migration of epithelial sheets is very sensitive to EFs and exhibits a collective galvanotactic response (Cohen, James Nelson et al. 2014).

Understanding how cells respond to EFs in vitro is informative but the in vivo dynamics of re-epithelialization of wounds in both wound healing and regenerative context is more applicable. Early studies were able to establish a correlation between disruptions of the wound induced EF and failure to re-epithelialize the wound (Stump and Robinson 1986, Rajnicek, Stump et al. 1988, Robinson Rhodes, Turek et al. 1990). The first study to clearly show that the current was responsible for driving re-epithelialization of the wound was performed in the red spotted newt. This study shows that when the wound
current is abolished, by either benzamil-mediated blockade of ENaC channels or exogenous application of an opposite and equal EF flowing into the wound, that the wound epithelium fails to form. Furthermore, re-establishment of the current in benzamil treated animals by exogenous application of a physiological EF restored wound healing capabilities only when the current flowed out of the wound (Chiang, Cragoe et al. 1991). This feature has also been demonstrated in corneal wound healing models where PI(3)Kγ and PTEN are critically involved in the cells ability to sense the EF and respond to it accordingly (Zhao, Song et al. 2006, Zhao 2009, Kucerova, Walczysko et al. 2011).
1.2.4 Other Cellular Level Influences of Electric Fields on Regeneration and Wound Healing

Two other cellular processes that are vital for tissue regeneration and wound healing could be influenced by the wound induced EF. The first of these is cellular proliferation. Much of the work done to establish electric fields as a stimulator of cellular proliferation have been conducted in vitro. The first of these studies were conducted on primary cultures of osteoblast-like cells cultured in the presence of low-amplitude AC EFs and resulted in increased rates of proliferation associated with increased alkaline phosphatase activity as well as increased expression of IGF-II, VEGF and ECM proteins (Fitzsimmons, Strong et al. 1992, Hartig, Joos et al. 2000, Kim, Song et al. 2006). Proliferation is also induced in an in vitro model of chronic wounds consisting of human dermally-derived fibroblast when cultured in an alternating current electric field. (Goldman and Pollack 1996, Cheng and Goldman 1998)

Differentiation or cell fate decisions of progenitor cells are an additional cellular function critical to regeneration and wound healing that could be influence by the wound induced EF. Cardiomyocyte differentiation is promoted in human embryonic stem cells when they are exposed to electrical stimulation in culture (Sauer, Rahimi et al. 1999, Serena, Figallo et al. 2009). This ability has recently been recapitulated in human induced pluripotent stem cells which could lead to
promising clinical therapies in regards to cardiac disease and myocardial infarction (Ma, Liang et al. 2016). Neural progenitors can be steered to either neuronal or astrocyte differentiation depending on the frequency of the alternating current EF in which they are cultured (Matos and Cicerone 2010). This phenomenon is not limited to excitable cells as osteogenic differentiation can be induced by culture in EF in both human adipose derived stem cells and bone marrow derived mesenchymal stem cells (McCullen, McQuilling et al. 2010, Hronik-Tupaj, Rice et al. 2011). These findings provide a good base of knowledge to support further investigation into the involvement of ionic EFs at regulating cellular proliferation and differentiation in vivo in the context of regeneration and wound healing.
1.2.5 How do Cells Sense and Respond to Electric Fields

Direction of cell migration throughout regenerating tissue is an essential aspect of regenerative and wound-healing processes (Hanna 1966). There is a long-standing hypothesis that endogenous ionic currents act to drive cell migration in development, wound healing, and regeneration (Jaffe and Nuccitelli 1977, Borgens, Vanable et al. 1979, Jaffe 1981). The mechanisms involved in this phenomenon are largely unknown but have garnered serious attention in recent years.

As previously stated, PI(3)Kγ and PTEN are known intracellular mediators of electrotactic migration in corneal epithelial cells but there still remains a gap in the understanding of how the cell senses the EF at the molecular level to stimulate intracellular signaling pathways (Zhao, Song et al. 2006). Figure 1.4 shows that the EF may direct migration through a combination of mechanisms (McCaig, Song et al. 2009). The green channels represent how electrophoretic forces can produce a concentration of channels and/or receptors on the leading edge of migrating cell. The EF could also induce an electrophoretic gradient of extracellular ligand concentration which would lead to a higher rate of leading edge channel activation even when channels are evenly distributed around the cell membrane (purple channels). Direct activation of voltage gated channels by modulation of leading edge membrane potential is also a possibility. Any of
these actions would lead to asymmetric intracellular signaling that could direct controlled cell migration (McCaig, Song et al. 2009).
1.2.6 Clinical Example of Electric Fields in Regeneration and Wound Healing

Given all of this data, it’s no surprise to find that there is an extensive record of using exogenous electrical stimulation (ES) in clinical settings to induce more rapid wound healing and neural regeneration. ES has been attributed to enhancing wound healing through a variety of mechanisms, including: stimulation of angiogenesis (Junger, Zuder et al. 1997) as well as tissue oxygenation in diabetic ulcers and lower extremity chronic ulcers of paraplegics (Kloth 2005). There have been a number of comprehensive reviews on clinical treatment of chronic wounds by ES and all of them reach the finding that ES stimulates wound healing and results in an increase in the rate of wound closure (Kloth and McCulloch 1996, Baker, Chambers et al. 1997, Gardner, Frantz et al. 1999, Kloth 2005). Clinical examples of the use of ES are not limited to the treatment of skin wounds and chronic ulcers. ES is an effective treatment to enhance both speed and precision of peripheral nervous system sensory and motor neuron axonal regeneration in traumatic injuries (Al-Majed, Neumann et al. 2000, Geremia, Gordon et al. 2007). The regenerative capacity of tissues in the central nervous system are far inferior to those of the peripheral nervous system, but spinal cord injuries respond well to ES, resulting in both functional recuperation and to a lesser extent, anatomical restoration of the lesion (Granat, Ferguson et al. 1993, Sadowsky 2001, Ragnarsson 2007, Hamid and Hayek 2008). These results are promising but a clearer understanding of the underlying physiology mediating these effects may lead to more effective treatments.
**Figure 1.1:** A) Representation of the formation of the trans-epithelial potential that acts as the battery for the wound induced electric field. B) Schematic drawing of the wound induced electric field. The field flows out through the center of the wounded area and the “battery” is recharged by the surrounding epithelium.
Figure 1.2: Molecular makeup involved in generating the trans-epithelial electric potential
Figure 1.3: Emil Du Bois Reymond using his homemade galvanometer to measure a wound induced current inflicted on himself. (du Bois-Reymond 1843, Finkelstein 2003)
<table>
<thead>
<tr>
<th><strong>Species</strong></th>
<th><strong>Body part</strong></th>
<th><strong>Duration</strong></th>
<th><strong>Current /EF</strong></th>
<th><strong>Reference</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Human</strong></td>
<td>Skin Wound</td>
<td></td>
<td>1 µA</td>
<td>(du Bois-Reymond 1843, Herlitzka 1910)</td>
</tr>
<tr>
<td><strong>Human</strong></td>
<td>Skin Wound</td>
<td></td>
<td>85 mV/mm</td>
<td>(Mukerjee, Isseroff et al. 2006)</td>
</tr>
<tr>
<td><strong>Human</strong></td>
<td>Skin Wound</td>
<td>3</td>
<td>~180 mV/mm</td>
<td>(Nuccitelli, Nuccitelli et al. 2008)</td>
</tr>
<tr>
<td><strong>Mouse</strong></td>
<td>Skin Wound</td>
<td></td>
<td>177 mV/mm</td>
<td>(Nuccitelli, Nuccitelli et al. 2008)</td>
</tr>
<tr>
<td><strong>Guinea Pig</strong></td>
<td>Skin Wound</td>
<td></td>
<td>100-200 mV/mm</td>
<td>(Barker, Jaffe et al. 1982)</td>
</tr>
<tr>
<td><strong>Bovine</strong></td>
<td>Corneal Epithelial Wound</td>
<td></td>
<td>42 mV/mm</td>
<td>(Chiang, Robinson et al. 1992, Sta Iglesia and Vanable 1998)</td>
</tr>
<tr>
<td><strong>Red-Spotted Newt</strong></td>
<td>Limb Stump</td>
<td>5-10</td>
<td>10-100 µA/cm²</td>
<td>(Borgens, Vanable et al. 1977, McGinnis and Vanable Jr 1986)</td>
</tr>
<tr>
<td><strong>Rana Pipiens (post metamorph)</strong></td>
<td>Limb Stump</td>
<td>~10 days</td>
<td>20-40 µA/cm²</td>
<td>(Borgens, Vanable et al. 1979)</td>
</tr>
<tr>
<td><strong>Red-Spotted Newt</strong></td>
<td>Digit Tip</td>
<td></td>
<td>42 mV/mm</td>
<td>(Chiang, Cragoe et al. 1989, Sta Iglesia, Cragoe et al. 1996)</td>
</tr>
<tr>
<td><strong>Human (child)</strong></td>
<td>Amputated Finger Tip</td>
<td></td>
<td>22 µA/cm²</td>
<td>(Illingworth and Barker 1980)</td>
</tr>
<tr>
<td><strong>Xenopus laevis</strong></td>
<td>Tail stump</td>
<td>2-4 hours</td>
<td>4.5 µA/cm²</td>
<td>(Reid, Song et al. 2009)</td>
</tr>
</tbody>
</table>

**Table 1.1** This table outlines wound induced currents that have been measured across different species with differing regenerative abilities.
Figure 1.4: A proposal for how cells may sense and migrate along a biologic electric field. Reprinted from: (McCaig, Song et al. 2009)
1.3 Ion Channel Regulation of Critical Cellular Dynamics

1.3.1 Ion Channels in Proliferation

1.3.1.1 Membrane potential during cell cycle progression

The cell cycle is a complex process that consist of phases that have many distinct characteristics (Braun-Dullaeus, Mann et al. 1998). These include fluctuation in cellular membrane potential \( V_{\text{mem}} \), ion channel expression, and membrane conductance. In stage \( G_0 \), cells are non-proliferative or in-between cycles and this is characterized by a more polarized \( V_{\text{mem}} \) which differs from cell type to cell type, but is on average -40 mV in non-excitable cells. Stage \( G_1 \) is characterized by cells that are preparing to enter the cell cycle. His stage is marked by increased conductance of voltage gated \( \text{Na}^+ \) and \( \text{Ca}^{2+} \) channels that result in an overall depolarization of \( V_{\text{mem}} \) to around -10 mV. Transition from \( G_0/G_1 \) phase into S phase can be blocked by pharmacological activation or blockade of voltage gated \( \text{K}^+ \) channels which indicates that this depolarization is critical for this process. S phase, characterized by DNA replication, is marked by reduced conductance of \( \text{Na}^+ \) and \( \text{Ca}^{2+} \) and increased conduction of voltage gated \( \text{K}^+ \) channels which results in repolarization of \( V_{\text{mem}} \) to -40 mV. The last phases of the cell cycle are \( G_2 \) and Mitosis which is characterized by cell shrinkage and cell division. Progression through these phases of the cell cycle are associated with
a final depolarization event that results from increased conductance of Na⁺, Cl⁻, Ca²⁺, and K⁺. The resultant efflux of Cl⁻ and K⁺ ions into the extracellular space are followed by water molecules leading to cell shrinkage and Cl⁻ channel blockade prevents transition from G2 phase to mitosis (Sachs, Stambrook et al. 1974, Blackiston, McLaughlin et al. 2009, Rao, Perez-Neut et al. 2015).

1.3.1.2 Ca²⁺ Signaling and its Role in Proliferation

Certainly, the most well understood class of ion sensitive regulatory networks are those that utilize Ca²⁺ as a second messenger (Berridge, Lipp et al. 2000). The idea that Ca²⁺ is involved in regulating cellular proliferation and progression through the cell cycle dates back almost 50 years and our understanding of this process has grown exponentially during that time (Balk 1971). The role of Ca²⁺ channels and Ca²⁺ conductance through the cellular membrane in establishing and maintaining V_mem was discussed in the previous section. This section will focus on the role of intracellular free Ca²⁺ concentrations and how this acts as a potent regulator of signaling cascades that drive progression through the cell cycle.

Ca²⁺ signals rely on a few main factors to translate intracellular concentrations of a relatively simple molecule into a discernable message able to drive a multitude
of intracellular and intercellular functions. The spatial property of the signal is the first major factor and this can range from a very localized rise in intracellular free Ca\(^{2+}\) to a global change that impact the entire cytoplasm. The timing of the signal is also important since sustained increases in intracellular free Ca\(^{2+}\) can be fatal to cell. This is overcome by using either low-amplitude signals or brief Ca\(^{2+}\) transients and sparks to stimulate acute cellular processes such as exocytosis or muscle contraction. On the other hand, repetitive rise and fall of intracellular concentrations known as Ca\(^{2+}\) oscillations are used to deliver information over the long term. Information is stored in the frequency of these oscillations and one of the most well-known effectors of this response is calmodulin-dependent protein kinase II (CAMKII) which will adjust its activity in response to changes in the frequency of Ca\(^{2+}\) oscillations. The amplitude of Ca\(^{2+}\) surges also hold information that can differentially activate unique signaling mechanisms as well, although the range of information stored in the amplitude of Ca\(^{2+}\) is not as abundant as what is stored in the frequency of the signal (Dolmetsch, Lewis et al. 1997, Berridge, Bootman et al. 1998).

One of the cellular behaviors that is sensitive to amplitude modulation of Ca\(^{2+}\) signals is cellular proliferation (El Boustany, Katsogiannou et al. 2010). There are several mechanisms of calcium entry that are relevant to its control of proliferation. The most common mechanism for increasing intracellular free Ca\(^{2+}\) in non-excitable cells is release from intracellular stores or Store-operated calcium entry (SOCE). This process starts with activation of plasma membrane
receptors that result in intracellular production of inositol 1,4,5-trisphosphate (IP$_3$) that in turn activates IP$_3$Rs to stimulate release of Ca$^{2+}$ from the endoplasmic reticulum (ER). Stromal interaction molecule 1 (STIM1) senses this depletion of ER Ca$^{2+}$ stores and interacts with Orai proteins and activate Ca$^{2+}$ release-activated channels (CRAC) to allow entry of Ca$^{2+}$ from the extracellular space effectively amplifying the initial calcium signal (El Boustany, Katsogiannou et al. 2010). Voltage-gated Ca$^{2+}$ channels are also expressed in non-excitable cells and are involved in proliferation with their permeability tied to the cell cycle phase dependent fluctuations in $V_{\text{mem}}$ discussed in a previous section (Takahashi, Yamaguchi et al. 1993). Elementary or localized calcium surges are confined to a small area within the cell and can be generated by either extracellular Ca$^{2+}$ or from intracellular Ca$^{2+}$ stores. The surrounding environment of the activated channels greatly influence the final outcome leading to terminal processes as diverse as muscle relaxation in one instance or muscle contraction in the other (Nelson, Cheng et al. 1995, Porter, Bonev et al. 1998). If these initial localized Ca$^{2+}$ surges are strong enough they can recruit other channels and affect intracellular free Ca$^{2+}$ concentrations at a global level and stimulate activity of Ca$^{2+}$ sensitive signaling pathways involving calmodulin, CAMKII and/or calcineurin (Berridge, Bootman et al. 1998).
1.3.1.3 Potassium Channels in Proliferation

Whereas changes in $V_{\text{mem}}$ during the generation of an action potential is very fast, $V_{\text{mem}}$ oscillations during the cell cycle are much slower with a cycle that last 10-20 hours depending on the cell type. Modulation of $K^+$ channel expression and function throughout the cell cycle has been known for some time (DeCoursey, Chandy et al. 1984, Takahashi, Yamaguchi et al. 1993). One of the main functions of $K$ channels in cell proliferation is driving the characteristic fluctuation in $V_{\text{mem}}$ associated with progression through the cell cycle. It has been proposed that this voltage-gated $K^+$ ($K_V$) channel function is one of the main influences that drive the essential $Ca^{2+}$ oscillations during the cell cycle (Lee, Sayeed et al. 1993). They are also important in the regulation of cell volume throughout the cell cycle (Takahashi, Yamaguchi et al. 1993). There are many cellular functions associated with cell cycle processes that require functional $K^+$ channel function to proceed normally.

Next we will discuss some of the more potent regulators of proliferation that we can identify down to the molecular level. $K_V1.3$ was one of the first $K^+$ channels identified as influencing proliferation in T lymphocytes and this result soon spread to other immune and progenitor cells including microglia, oligodendrocytes, and
macrophages (DeCoursey, Chandy et al. 1984, Kotecha and Schlichter 1999, Chittajallu, Chen et al. 2002, Vicente, Escalada et al. 2003, Fordyce, Jagasia et al. 2005, Villalonga, David et al. 2010). \(K_v11.1\) is another channel identified as involved in regulating cellular proliferation with its expression upregulated in many cancer lines where it appears to be responsible for the oncogenic stimulation of proliferation (Afrasiabi, Hietamäki et al. 2010, Glassmeier, Hempel et al. 2012). \(K_v10.1\) is a closely related channel to \(K_v11.1\) discussed above and it also seems to be oncogenic in response to overexpression via increased stimulation of proliferation in many cancer types, such as leukemia, ovarian, and colon cancer although we don’t have a full picture of the mechanisms underlying this role (Pardo, del Camino et al. 1999, Ousingsawat, Spitzner et al. 2007, Agarwal, Griesinger et al. 2010, Asher, Khan et al. 2010).

1.3.1.4 Chloride Channels in Proliferation

Little is known about how chloride channels regulate cellular proliferation but data has been picking up over the past decade. Varela and colleagues have shown that application of \(H_2O_2\) (an important early signaling molecule in regeneration) activates volume-sensitive chloride channels and in turn leads to an induction of cell proliferation (Varela, Simon et al. 2004, Love, Chen et al. 2013). Phipps’ study from 1996 indicated that broad scale blockade of \(Cl^-\) channels with 4,4'-Diisothiocyanatostilbene-2,2'-disulfonic acid (DIDS) and 3 other blockers inhibited
the activation and signaling of T-lymphocytes through a modulation of Ca\(^{2+}\) signaling (Phipps, Branch et al. 1996). Additionally, Wondergem and colleagues show that blockade of chloride channels with a variety of chloride channel blockers, including DIDS, reduces proliferation in cultured mouse liver cells (Wondergem, Gong et al. 2001). Much of this work to date has focused on the role of chloride channels in cell volume expansion and contraction throughout the cycle which is at least partially dependent on functional membrane chloride conductance (Chen, Zhu et al. 2007, Xu, Mao et al. 2010). Since the recent discovery of Ano1/2 as the molecular determinants of the Ca\(^{2+}\)-activated Cl\(^{-}\) current, they have garnered much interest as regulators of proliferation (Wang, Yang et al. 2012, Britschgi, Bill et al. 2013). It is thought that they act through MAPK, EGFR and CAMK in different biological contexts, although the intermediary mechanisms have remained elusive (Duvvuri, Shiweis et al. 2012, Wang, Yang et al. 2012, Britschgi, Bill et al. 2013)
A variety of ion channels have been indicated as required for \textit{in vitro} cell migration in multiple cell types such as: N-methyl-D-aspartate receptor (ligand-gated), Ca\(^{2+}\) permeable ion channel (Li, El-Hayek et al. 2008), Calcium dependent potassium channel (\(K_{Ca}\)) 3.1 (Cruse, Duffy et al. 2006), voltage-gated potassium channel (\(K_v\)) 1.1 (with a similar response elicited by removal of extracellular \(Ca^{2+}\)) (Wang, Wang et al. 2000), epithelial sodium channels (ENaC) (Kapoor, Bartoszewski et al. 2009), and voltage gated sodium channel (\(Na_v\)) 1.7 (Meguro, Iida et al. 2009). The migratory effects of EFs have also been shown to override the directional influence of other migratory cues present during wound healing (Zhao, Song et al. 2006). It is reasonable to assume that some of these channels are activated downstream of direct interaction with the EF which leads to the following questions: 1) What are the specific interactions between EFs and cell membrane proteins that direct migration? 2) Are these interactions responsible for regeneration’s dependence on EF?
Rationale, Overall Hypothesis and Specific Aims

This study plans to elucidate specific ion channels that are influenced by EF to direct critical cellular dynamics as it relates to the EF dependence in regeneration and to understand the conserved molecular mechanisms that may persist in biological systems of higher vertebrates. The proposed experiments, outlined below, will allow for a better understanding of cellular control mechanisms in a naturally existing model of vertebrate regeneration and then move on to specific signaling mechanisms that may be conserved in mammalian cells. These are critical steps to developing clinical applications and advancing regenerative medicine to the next level. Specifically, the objective of this work is to elucidate the signaling mechanisms involved in transducing the signal from activated ion channels into a cellular response. This objective is based on a literature discussed above indicating a role for ion channels and bioelectric signaling in control of critical cellular processes in response to wounding. The central hypothesis to be tested is that ion channels are activated in response to wounding and act to either induce cellular level responses or create a permissive environment for proliferation, migration and differentiation in critical cell populations required for wound healing and regeneration. The long-term goal of this work is to identify molecules and signaling pathways that are critical for driving proliferation in regeneration and wound healing and to understand the mechanisms that constrain regenerative capacity in higher vertebrates. This work will be laid out in the following specific aims:
Specific Aim 1 investigated soft tissue cellular response of immune cells to exogenously applied electric fields of physiological magnitude and gauge the involvement of ion channels in mediating these effects

- To accomplish this I evaluated in vivo cellular-level response of immune cells to exogenous sine wave stimulation of tail muscle in the crayfish Procambarus clarkii
- Also, I evaluated the role of voltage-gated and inward rectifying potassium channels in mediating these observed effects

Specific Aim 2 elucidated the molecular level determinants of the influence of ion channels on amphibian tail regeneration

- To accomplish this I performed a chemical genetic screen of ion channel antagonist against axolotl embryo tail regeneration to identify individual ion channels involved in mediating the bioelectric influence on regeneration.

Specific Aim 3 evaluated the cellular level influences of individual ion channels identified by the chemical genetic screen in aim 2

- To accomplish this I evaluated progenitor cell proliferation in regenerating axolotl embryos in response to pharmacological inhibition of select ion channels
- Also, I evaluated immune cell activation in regenerating axolotl embryos in response to pharmacological inhibition of select ion channels
- Finally, I evaluated candidate gene expression of signaling pathways suspected to be influenced by identified ion channels using quantitative PCR

Specific Aim 4 evaluated the conservation of anoctamin 1 mediated regulation of proliferation in mammalian cells and identify the molecular mechanism that drives it.

- To accomplish this I evaluated calcium signaling in HEK293 cells using pharmacological inhibition, genetic knockout and overexpression of anoctamin 1 using fluorescent indicators of intracellular Ca^{2+} concentrations
• Also, I evaluated Erk1/2 signaling activation in response to pharmacological inhibition and genetic knock out of anoctamin 1 in HEK293 cells
• Finally, I evaluated Ca^{2+} cellular proliferation in HEK293 cells in response to pharmacological inhibition and genetic knock out of anoctamin 1
CHAPTER 2

SINE WAVE ELECTRICAL STIMULATION INITIATES A VOLTAGE-GATED POTASSIUM CHANNEL DEPENDENT SOFT TISSUE RESPONSE CHARACTERIZED BY INDUCTION OF HEMOCYTE RECRUITMENT AND COLLAGEN DEPOSITION

Brandon Michael Franklin, Eleni Maroudas and Jeffrey L. Osborn

Abbreviations used: ES (electrical stimulation), Kv (voltage-gated potassium channel), Ca^{2+} (calcium cation), EF (electric field), K^+ (potassium cation), LA (Louisiana), mm (millimeter), mV (millivolt), Hz (hertz), ms (milliseconds), µl (microliter), PBS (phosphate buffered saline), H&E (hematoxylin and eosin), min (minute), 4-AP (4-aminopyridine), µM (micro molar), AZ (astemizole), RR (ruthenium red), µm (micrometer), ANOVA (analysis of variance), SNK (Student-Newman-Keuls), GC (granulocyte), SGC (semi-granulocyte), HY (hyaline hemocyte), EAG (ether a-go-go), hEAG (human ether a-go-go), CFTR (cystic fibrosis transmembrane receptor), CLIC (chloride intracellular channel)

Keywords: Electrical stimulation, hemocyte, macrophage, voltage-gated potassium channels.

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2.1 Abstract

Soft tissue repair is a complex process that requires specific communication between multiple cell types to orchestrate effective restoration of physiological functions. Macrophages play a critical role in this wound healing process beginning at the onset of tissue injury. Understanding the signaling mechanisms involved in macrophage recruitment to the wound site is an essential step for developing more effective clinical therapies. Macrophages are known to respond to electrical fields, but the underlying cellular mechanisms mediating this response is unknown. This study demonstrated that low amplitude sine-wave electrical stimulation (ES) initiates a soft tissue response in the absence of injury in *Procambarus clarkii*. This cellular response was characterized by recruitment of macrophage-like hemocytes to the stimulation site indicated by increased hemocyte density at the site. ES also increased tissue collagen deposition compared to sham treatment (p<0.05). Voltage-gated potassium (Kv) channel inhibition with either 4-aminopyridine or astemizole decreased both hemocyte recruitment and collagen deposition compared to saline infusion (p<0.05) whereas inhibition of calcium-permeable channels with ruthenium red did not affect either response to ES. Thus, macrophage-like hemocytes in P. clarkii elicit a wound-like response to exogenous ES and this is accompanied by collagen deposition. This response is mediated by Kv channels but independent of Ca\(^{2+}\) channels. We propose a significant role for Kv channels that extends
beyond facilitating Ca$^{2+}$ transport via regulation of cellular membrane potentials during ES of soft tissue.
2.2 Introduction

Repair of soft tissue damage is a critical need of living organisms that involves basic restoration of anatomical structures and functions to damaged tissue. This is a delicate process and its execution is not always optimal, as either excessive healing (e.g. fibrosis and adhesions) or inadequate healing (e.g. chronic wounds and ulcers) can lead to diminished or lacking restoration of function (Lazarus, Cooper et al. 1994, Diegelmann and Evans 2004). An essential mediator of the wound repair process is the infiltration of the wound region with macrophages (Clark 2013). Disruption of normal macrophage activity in wound healing can contribute to decreased inflammatory cytokines, neutrophil removal, angiogenesis, fibroblast proliferation and collagen deposition (Gardner, Frantz et al. 1999, Mirza, DiPietro et al. 2009, Koh and DiPietro 2011, Clark 2013). Conversely, activation or introduction of macrophages at the site of the wound accelerates wound healing in experimental models of impaired wound healing (Danon, Kowatch et al. 1989, Chen, Tredget et al. 2008). Wound induced electrical fields (EFs) are an intrinsic property of damaged tissues that are vital to the wound healing process across different species (Chiang, Cragoe et al. 1991, Jenkins, Duerstock et al. 1996, Reid, Song et al. 2005, Wang and Zhao 2010, Messerli and Graham 2011). Also, macrophages are sensitive to electrical fields and this property may be somewhat responsible for the positive effects of electrical stimulation (ES) on wound healing and repair of neural tissue (Moriarty and Borgens 1998, Cho, Thatte et al. 2000).
These findings suggest that wound induced EFs direct macrophage migration to
the wound site but few studies identify the mechanisms responsible for facilitating
this action. There are long standing hypotheses that endogenous ionic currents
act to control cell dynamics in development, wound healing and regeneration
(Jaffe and Nuccitelli 1977, Borgens, Vanable et al. 1979, Jaffe 1981, Ozkucur,
Epperlein et al. 2010). However, the mechanisms utilized by cells to detect the
EF and translate it into a discernable message to drive specific cell behaviors,
such as migration, proliferation and differentiation, are not well understood. A
better understanding of how cells are able to sense EFs and react to them is vital
to understanding the global physiology involved in tissue repair. Ion channel
signaling provides a reasonable cellular response for mediating these effects
based on their documented involvement in cell proliferation, migration and
differentiation (Lang, Foller et al. 2005, Prevarskaya, Skryma et al. 2007,
Schwab, Fabian et al. 2012). Specifically, voltage-gated and calcium-sensitive
potassium channels are involved in regulating a variety of macrophage functions
including activation, migration and cytokine secretion (Gallin 1984, Mackenzie,
Chirakkal et al. 2003, Dong, Ji et al. 2013).

The present study investigated this phenomenon using an invertebrate
homologue of macrophages, crustacean hemocytes, by measuring how they
respond to exogenous ES and how this response is mediated by both potassium
and calcium channel signaling. The response to ES was assessed using basic
histological techniques and pharmacological antagonists were used to assess the role of $K^+$ and $Ca^{2+}$ channels in hemocyte recruitment during ES.
2.3 Materials and Methods

2.3.1 Animal preparation

Adult *Procambarus clarkii* ranging from 3-5 inches in length were procured from Atchafalay Biological in Raceland, LA and allowed to acclimate to the laboratory aquatic living environment for at least two weeks. Animals that were in the process of molting or had recently molted were excluded from the study. Animals selected for this study were anesthetized in ice water for thirty minutes before being instrumented for the experiment. A small hole (2 mm X 2 mm) was made in the third segment of the dorsal tail carapace. Stainless steel electrode tips (4 mm x 2 mm) were implanted between the carapace and the tail muscle surface with the cathode on the left and the anode on the right. For experiments requiring infusion of pharmacological agents or vehicle, polyethylene tubing connected to an infusion pump was inserted into the same hole as the cathode ([Figure 2.1](#)). Carapace opening closure and securment of the instrumentation was conducted using fast drying cyanoacrylate (commercial superglue) applied to the openings. Animals then were placed in individual water-filled enclosures and allowed 2-4 recovery days before beginning the experiment.
2.3.2 Treatment protocol

Following the four day recovery period, drug or vehicle infusion was initiated. Acclimation for 24 hours was allowed to ensure total blockade of targeted ion channels before initiating electrical stimulation. Tail muscles were intermittently stimulated with sine wave impulses at 450 mV and 2 Hz (50ms on to 450ms off) continuously for four days. Immediately following the four-day stimulation period, animals were anesthetized in ice water and euthanized by decapitation. Evans Blue dye was injected (10 µl) through the electrode opening of the carapace to mark the site of electrode implantation. The tail muscle then was exposed and a ~20mm cross section containing the tissue subjected to ES was excised. Tail muscle was fixed by submersion in 4% paraformaldehyde for 12-18 hours. Tissues were rinsed in PBS and cyoprotected in 15% sucrose for four hours followed by 30% sucrose overnight, frozen and stored at -20°C. Tissues were embedded in OTC compound and cut into 15 micron tissue sections by cryostat sectioning and mounted onto gelatin coated slides. Tissue sections were stained with hematoxylin and eosin (H&E), Masson's trichrome, or picrosirius red according to standard protocols.

2.3.3 Ion channel inhibition

To examine the role of potassium and calcium channels, pharmacological antagonists were solubilized in standard crayfish saline and infused at 1.25
µl/min before and during the ES. To block voltage-gated potassium ($K_V$) currents, 4-Aminopyridine (4-AP) was infused (1.25 µl/min) at a concentration of 10 µM and Astemizole (AZ) was infused (1.25 µl/min) at a concentration of 5 µM. Calcium ($Ca^{2+}$) signaling was investigated by infusion of ruthenium red (RR) at 10 µM (Hirano, Imaizumi et al. 1998, Taglialatela, Pannaccione et al. 1998, Nattel, Matthews et al. 2000, Clapham, Runnels et al. 2001, Wulff, Castle et al. 2009). Crayfish saline was infused as vehicle control. Infusion commenced 24 hours prior to initiation of the experiment to ensure the entire tail muscle was thoroughly bathed in each compound preceding ES onset. When infusion was initiated, crayfish were observed out of their environments for 20 min to ensure there was no leakage of the infusate around the surgical sites or other areas. In total, there were 5 treatment groups: Sham electrode implantation + saline infusion (Sham/Saline), ES electrodes + saline infusion (Stim/Saline), ES electrodes + 4-AP infusion (Stim/4-AP), ES electrodes + AZ infusion (Stim/AZ), and ES electrodes + RR infusion (Stim/RR). All groups were n=6.

### 2.3.4 Analysis of hemocyte activation and collagen deposition

To measure the level of hemocyte activation, histological H & E stained sections were examined. Hemocytes within 500 µm of the electrode implantation site were morphologically identified and assigned to one of three groups: granulocytes, semigranulocytes and hyaline cells. Hyaline hemocytes are characterized by their relatively small size, elongated oval shape, a centralized
nucleus, high nuclear/cytoplasmic ratio and an absence of cytoplasmic granules. Granulocytes are larger with an eccentric, oblong nucleus, lower nuclear/cytoplasmic ratio and an abundance of eosinophilic granules in the cytoplasm. Semigranulocytes are similar in size and nuclear/cytoplasmic ratio to granulocytes with an eccentric, spherical nucleus and a reduced number of eosinophilic granules in the cytoplasm (Lanz, Tsutsumi et al. 1993, Parrinello, Sanfratello et al. 2015). Each group then was normalized to tissue section area. Values are expressed as hemocytes per 10,000 µm². Collagen deposition was assayed both qualitatively and semi-quantitatively. Qualitative evaluation was achieved with Masson’s trichrome staining producing blue collagen, red cytoplasm/muscle fiber and black nuclei. Semi-quantitative assessment was achieved by measuring the percentage of tissue area, within 500µm (a total tissue area of 0.5 mm²) of electrode implantation, staining positive for collagen by polarized light imaging of picrosirius red stained tissue sections.

2.3.5 Statistical Analysis

Statistical differences between or among groups were determined using student’s t-test or one-way ANOVA. Post hoc analysis was conducted using a Student-Newman-Keuls (SNK) method to determine statistical differences between mean values for each treatment and the control group. All data are presented as mean ± standard deviation. The 0.05 level of probability was utilized as the criterion for significance in all data sets.
Figure 2.1: (A) Schematic drawing of the experimental preparation. Electric stimulation is supplied by the signal generator and pharmacological inhibitors are delivered via a syringe infusion pump. (B) Photographic representation of an instrumented crayfish.
2.4 Results

2.4.1. Intermittent sine-wave ES elicits hemocyte activation and collagen deposition in crayfish tail muscle

Following continuous ES over a 4-day period, the tissue area directly under the site of electrode implantation exhibited an aggregation of hemocytes analogous to the first stage of the wound healing response as described in both penaeid shrimp and freshwater crayfish (Fontaine and Lightner 1973, Fontaine 1975). This hemocyte aggregation was not observed in sham treated animals without ES (Figure 2.2). Total hemocyte density in animals exposed to exogenous ES was increased (60.29±23.73 hemocytes/10,000µm²) compared to sham-treated animals (1.87±0.59 hemocytes/10,000µm², p<0.05), but this effect was not specific to one hemocyte subtype population (Figure 2.2).

Another defining characteristic of the crayfish wound response is the deposition of collagen and subsequent tissue fibrosis (Fontaine and Lightner 1973, Fontaine 1975). Separate histological techniques (Masson’s trichrome and picrosirius red staining) were employed to assay for collagen deposition and scarring. Masson’s trichrome stain identifies tissue fibrosis and collagen deposition by red cytoplasm, black nuclei and blue collagen fibers. Sham-treated animals had minimal collagen deposition adjacent to the electrode implantation site (Figure 2.3A). In contrast, animals exposed to exogenous ES exhibited significant
collagen deposition directly under the site of electrode implantation (Figure 2.3B). These tissues were also stained with picrosirius red and imaged under both bright field (yellow cytoplasm and red collagen) for qualitative assessment and polarized light (yellow-orange birefringence for thick collagen fibers and green birefringence for thin collagen fibers) for a semi-quantitative measurement of total collagen in the tissue adjacent to the site of electrode implantation.

**Figure 2.3C and 2.3D** depict representative sham-treated (C) and ES (D) animals. Significant collagen deposition is observed in ES animals but not in sham-treated animals. Using the picrosirius red images taken under polarized light, the percent area of fibrosis was measured. Exogenously stimulated animals exhibited fibrosis in a higher percentage (16.35±5.20%) of tissue adjacent to the electrode implantation site compared with sham-treated animals (1.47±1.03%, **Figure 2.4**; p<0.05).

**2.4 ES mediated hemocyte activation is dependent on Kv channels**

To assess the role of voltage-dependent potassium currents in mediating the response to ES, pharmacological modulators were infused continuously (1.25 µl/min) from 24 hours before initiation of ES to the end of the experiment. Blockade of Kv channels with either 4-AP (10.53±6.65 hemocytes/10,000 µm²) or astemizole (8.64±3.89 hemocytes/10,000 µm²) decreased the total hemocyte response to ES when compared to saline infusion (60.29±23.73 hemocytes/10,000 µm², p<0.05). This effect was not limited to any specific
hemocyte subtype population (Figure 2.4). Collagen deposition was also attenuated by either 4-AP (3.71±0.86%) or astemizole (4.27±1.87%) when compared with saline infused animals (16.35±5.20%, Figure 2.5, p<0.05).

2.4.3 Ca²⁺ channels are not required for the ES mediated response

Blockade of Ca²⁺ signaling with ruthenium red (RR) did not affect the hemocyte response to ES (46.38±16.81 hemocytes/10,000 µm²) compared to saline infused animals (60.29±23.73 hemocytes/10,000 µm², p>0.05; Figure 2.4). Collagen deposition also was not changed by RR (11.68±3.84%) when compared with saline infused animals (16.35±5.20%, p>0.05; Figure 2.5).
Figure 2.2: Hematoxylin and Eosin stained tissue sections of sham-treated (A) and stimulated (B) tail muscle (electrode placement depicted by red dash). An aggregation of hemocytes is observed directly beneath the site of electrode implantation in stimulated (n=6) but not sham treated (n=6) animals. Higher magnification of the subject depicted in panels C&D show this hemocyte aggregation in detail (GC-granulocyte, SGC-semigranulocyte, HY-Hyaline hemocyte). Hemocyte density near the electrode site was used as a measure of hemocyte activation. (E) Hemocyte density was increased in ES animals (n=6) compared to sham treated (n=6) animals. (F) This response was observed in all hemocyte subtypes (* = p<0.05 compared to sham/saline; student’s t-test)
Figure 2.3: Tissue sections were stained with Masson’s trichrome stain (A & B), picrosirus red (PR) under bright-field (C & D) and PR under polarized light (E & F). Electrically stimulated animals (B, D & F; n=6) exhibited increased collagen staining compared to sham-treated animals (A, C & F; n=6). A less enlarged micrograph of PR stained sham treated (G) and stimulated (H) tail muscle sections depict the overall morphology of the effect. (I) Total collagen was quantified by optical density of PR stained tissue sections imaged under polarized light. Values represent the percentage of a 0.5mm² area (white box) that stained positive for collagen. (* = p<0.05 compared to sham/saline; student’s t-test)
Figure 2.4: Hematoxylin and Eosin stained tissue sections of sham-treated/saline infused (A, n=6), stimulated/saline infused (B, n=6), stimulated/RR infused (C, n=4), stimulated/4-AP infused (D, n=5) and stimulated/AZ infused tail muscle (E, n=5; electrode placement depicted by red dash). (F) The hemocyte response was attenuated with blockade of Kv11.1 with either astemizole or 4-aminopyridine. Blockade of TRP Ca^{2+} channels with ruthenium red did not significantly alter the hemocyte response. (G) The influence of Kv channel blockade is not restricted to any of the hemocyte subtypes. (\* = p<0.05 compared to sham/saline; one-way ANOVA with SNK post hoc)
Figure 2.5: Picrosirus red stained tissue sections of sham-treated/saline infused (A, n=6), stimulated/saline infused (B&F, n=6), stimulated/RR infused (C&G, n=4), stimulated/4-AP infused (D&H, n=5) and stimulated/AZ infused tail muscle (E&I, n=5; electrode placement depicted by red dash). Collagen deposition was reduced by potassium channel blockade by both 4-aminopyridine (H) and astemizole (I) but was not affected by Ca^2+ cannel blockade with ruthenium red (G) compared to sham treated animals (F). (J) Total collagen (% of 0.5 mm^2 tissue section area; black box) was significantly reduced with blockade of Kv11.1 with either astemizole or 4-aminopyridine but not by blockade of TRP.
2.5 Discussion

The tissue response to low-amplitude, low-frequency sine-wave ES in the tail muscle of adult *P. clarkii* was characterized and compared to documented crustacean wounding responses (Fontaine and Lightner 1973, Fontaine 1975). The response of crayfish hemocytes was of particular interest considering the distinct similarities hemocytes share with macrophages and the documented role of macrophages in vertebrate wound healing (Danon, Kowatch et al. 1989, Hose, Martin et al. 1990, Moriarty and Borgens 1998, Söderhäll, Bangyeekhun et al. 2003, Chen, Tredget et al. 2008, Koh and DiPietro 2011). Secondly, the study identified Kv channels as one of the molecular determinants responsible for interpreting the electrical signal into a discernable message to direct cell activity.

Several studies have indicated that wound healing is dependent on a wound-induced electrical field and that this electrical field can be modulated by exogenous ES (Chiang, Cragoe et al. 1991, Jenkins, Duerstock et al. 1996, Wang and Zhao 2010, Messerli and Graham 2011). This study demonstrated that exogenous ES is sufficient to elicit a soft tissue response in *P. clarkii* tail muscle in the absence of soft tissue injury characterized by hemocyte accumulation and collagen deposition. Hemocyte/macrophage populations have been indicated as essential and early participants in the wound healing process. They play important roles in matrix degradation at the wound site, phagocytosis to remove debris and cytokine secretion to attract other important cell types
(Danon, Kowatch et al. 1989, Montagnani, Le Roux et al. 2001, Brancato and Albina 2011, Koh and DiPietro 2011, Clark 2013). In this study, an aggregation of hemocytes was observed in response to ES indicating that this exogenous electrical field stimulates hemocyte recruitment as would happen early on in the wound response. This is not surprising, as in vitro and in vivo studies have shown that macrophages respond to ES. Whether or not ES is directly acting on hemocytes or influencing the action of other cells that cause hemocyte infiltration is unclear but in vitro studies have established a relationship between macrophages and EFs. (Orida and Feldman 1982, Cho, Thatte et al. 2000, Hoare, Rajnicek et al. 2015)

Another typical and consistent characteristic of a wound response is the deposition of collagen (Clark 2013). As shown in figure 2.3, the application of ES led to significant collagen deposition. Previously characterized models of crustacean wound responses in penaeid shrimp and P. clarkii describe a similar histological response to what has been described in these data (Fontaine and Lightner 1973, Fontaine 1975). These results indicate that low-amplitude, low-frequency sine wave ES of the crayfish tail muscle brings about some of the typical characteristics of crayfish wound healing in the absence of tissue insult. This provides a basis and rationale for the in vivo study of the molecular mechanisms involved in wound-induced EF mediated repair processes.
Considering the documented role of potassium channels in both wound healing and macrophage activity, it was reasonable to suspect that they had a role in interpreting the electrical signal to elicit the results seen in this study (Gallin 1984, Blunck, Scheel et al. 2001, Shin, Liu et al. 2002, Anděrová, Antonova et al. 2004, Kan, Gao et al. 2016). In other studies, Kv channels have been shown to regulate proliferation in multiple tumor cell lines, with strong evidence specifically indicating Kv10.1 and Kv11.1 (EAG and hEAG) channels (Bianchi, Wible et al. 1998, Conti 2004). Multiple cell types have demonstrated a dependence on K+ channel signaling for proper direction of cell migration (Schwab and Oberleithner 1995, Da Silva-Santos, Santos-Silva et al. 2002, Dal-Secco, Cunha et al. 2008, Jin, McCloskey et al. 2008, Silver, Littlejohn et al. 2015). The fact that collagen synthesis and deposition is reduced is not surprising, considering that previous studies have found that macrophages stimulate collagen synthesis and scar formation and that specific ablation of macrophage populations before wounding results in reduced collagen deposition (Hunt, Knighton et al. 1984, Portera, Love et al. 1997, Mirza, DiPietro et al. 2009).

The data fall short of revealing the exact mechanism of Kv channels’ involvement in this response and Kv blockade does not completely inhibit the response. ES has been shown to manipulate multiple cellular behaviors including migration, proliferation and cytokine production and it could be that Kv are only involved in some of these processes. (Fitzsimmons, Strong et al. 1992, Li and Kolega 2002, Wang, Yin et al. 2003, Kim, Song et al. 2009, Zhao 2009) Further research is
needed to understand if Kv impacts hemocyte proliferation, migration, cytokine secretion or some other mechanism in the context of wound healing. This study has shown that hemocyte infiltration can be induced via ES and that this model of ES induced hemocyte infiltration also produced collagen deposition. Although, when hemocyte infiltration is blocked, ES alone is not sufficient to induce collagen deposition.

Ca^{2+} permeable channels have been shown to be critical regulators of cell function and are sensitive to potassium channel signaling (Lallet-Daher, Roudbaraki et al. 2009, Billeter, Hellmann et al. 2014, Schilling, Miralles et al. 2014). The classic Ca^{2+} channel inhibitor ruthenium red had no effect on either hemocyte activation or collagen deposition (Figures 2.4 and 2.5). This indicates that Ca^{2+} channel signaling is not a required mediator of the response to ES, although ruling out a complete role for Ca^{2+} signaling in facilitating this response may be premature. Chloride channels were not investigated in this study but many of them (CFTR, glycine-gated and CLICs) have been indicated as regulators of macrophage and other immune cell function via phagosomal acidification, cytokine production, Ca^{2+} influx and superoxide production (Ikejima, Qu et al. 1997, Wheeler and Thurman 1999, WHEELER, STACHLEWITZ et al. 2000, Di, Brown et al. 2006, Jiang, Salao et al. 2012). The results of the present study clearly indicate that exogenous ES induces a response characterized by hemocyte aggregation and collagen deposition that closely resembles a
documented crustacean wound response and that Kv channels are a critical component of this response (Fontaine and Lightner 1973, Fontaine 1975).
CHAPTER 3

ION CHANNEL SIGNALING INFLUENCES CELLULAR PROLIFERATION AND PHAGOCYTE ACTIVITY DURING AXOLOTL TAIL REGENERATION

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Abbreviations used: $K_V$ (voltage-gated potassium channel), $Ca_V$ (Voltage-gated calcium channel), GlyR (glycine receptor), GABA$_A$R (GABA type A receptors), SERCA (sarcoplasmic/endoplasmic reticulum calcium ATPase), MAPK (mitogen-activated protein kinase), erk (extracellular signal regulated kinase), $H^+$ (hydrogen cation or proton), EF (electric field), mm (millimeter), IACUC (institutional animal care and use committee), BTS (N-benzyl-p-toluene sulfonamide), mM (millimolar), NaCl (sodium chloride), KCl (potassium chloride), CaCl$_2$ (calcium dichloride), NaHCO$_3$ (sodium bicarbonate), min (minute), mL (milliliter), M (molar), dpa (days post amputation), SPSS (sigma plot statistical software), ANOVA (analysis of variance), μg (microgram), hpa (hours post amputation), hrs (hours), EdU (5-ethynyl-2'-deoxyuridine), EtOH (ethanol), DNA (deoxyribonucleic acid), TUNEL (Terminal deoxynucleotidyl transferase dUTP nick end labeling), μm (micrometers), SNK (Student-Newman-Keuls), RNA (ribonucleic acid), PCR (polymerase chain reaction), $C_6H_7NaO_7$ (monosodium citrate), EDTA (Ethylenediaminetetraacetic acid), (NH$_4$)$_2$SO$_4$ (ammonium sulfate), pH (potential of hydrogen), mRNA (messenger RNA), cDNA (complementary DNA), μM (micromolar), GAPDH (Glycerol-3-phosphate dehydrogenase), PKCy (protein kinase C gamma), PKCa (protein kinase C alpha), PKCβ1 (protein kinase C beta 1), RRAS (related RAS viral oncogene homolog), RAF1 (Raf-1 Proto-Oncogene, Serine/Threonine Kinase), MAPKK1 (MAPK kinase 1), MAPKK2 (MAPK kinase 2), MAPK1/ERK2 (mitogen-activated protein kinase 1 or extracellular signal regulated kinase 2), MAPK3/ERK1 (mitogen-activated protein kinase 3 or extracellular signal regulated kinase 1), DIDS (4,4'-Diisothiocyanatostilbene-2,2'-disulfonic acid), DNDS (4,4' dinitrostilbene-2,2'-disulfonic acid), NFA (niflumic acid), Cl$^-$ (chloride anion), CaCC (calcium activated chloride channel), Ano1 (anoctamin 1), Ano2 (anoctamin 2), BBM (benzbromarone), A01 (T16a(inh)-A01), PTx (picrotoxin), BCU (bicuculline methiodide), TEA (tetraethylammonium), 4-AP (4-aminopyridine), GTx-1E (Guangxitoxin-1E), CytB (cytochalasin B), ENaC (epithelial sodium channel), Nav (voltage-gated sodium channel), TTx (tetrodotoxin), BPD (bepridil), BNP (benidipine HCl), nM (nanomolar), tBuBHQ (2,5-di-(tert-butyl)-1,4-benzohydroquinone), NR (neutral red), hba (hours before amputation), ED (epidermal), SC (spinal cord), MM (muscle and mesenchymal), mek1 (MAPKK1), mek2 (MAPKK2), MSC (mesenchymal stem cells), ER (endoplasmic reticulum),
CNS central nervous system), AGSC (Ambystoma genetic stock center), NSF (national science foundation),

**Keywords:** regeneration, tissue repair, ion channels, bioelectric fields, proliferation, macrophages, calcium activated chloride channels, voltage-gated potassium channels, anoctamin 1

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3.1 Abstract

Little is known about the potential for ion channels to regulate cellular behaviors during tissue regeneration. Here, we utilized an amphibian tail regeneration assay coupled with a chemical genetic screen to identify ion channel antagonists that altered critical cellular processes during regeneration. Inhibition of multiple ion channels either partially (anoctamin1/Tmem16a, anoctamin2/Tmem16b, Kv2.1, Kv2.2, L-type Cav channels and H/K ATPases) or completely (GlyR, GABA\textsubscript{\textalpha}R, K\textsubscript{\textalpha}V1.5 and SERCA pumps) inhibited tail regeneration. Partial inhibition of tail regeneration by blocking the calcium activated chloride channels, anoctamin1&2, was associated with a reduction of cellular proliferation in tail muscle and mesenchymal regions. Inhibition of anoctamin 1/2 also altered the post-amputation transcriptional response of p44/42 MAPK signaling pathway genes, including decreased expression of erk1/erk2. We also found that complete inhibition via K\textsubscript{\textalpha} channel blockade was associated with diminished phagocyte recruitment to the amputation site. The identification of H\textsuperscript{+} pumps as required for axolotl tail regeneration supports findings in Xenopus and Planarian models, and more generally, the conservation of ion channel regulators of tissue regeneration. This study provides a preliminary framework for an in depth investigation of the mechanistic role of ion channels and their potential involvement in regulating cellular proliferation and other processes essential to wound healing, appendage regeneration and tissue repair.
3.2 Introduction

Ion channels are known for traditional physiological functions, including muscle contraction, nerve conduction and maintenance of ionic homeostasis. However, ion channels modulate membrane ion conductance across all cells and tissues, establishing electrical fields (EF) that affect cellular behaviors under normal conditions, during critical periods of development, and in response to tissue injury. Understanding how ion channels function within different biological contexts is central to identifying the molecular basis of channelopathies and for exploiting ion channels for wound healing and tissue repair. Bioelectric signaling via ion channels and control of cellular membrane potentials in planarian and *Xenopus* regeneration have significantly contributed to our understanding of ionic influences on regenerative processes (Levin 2007, Levin 2009, Tseng, Beane et al. 2010, Levin 2014). Despite these advances, little is known about the individual channels that are important during regeneration and the specific cellular functions that they influence.

Amphibians and fish provide powerful models to investigate the role of EF and ion channels on cellular processes that are activated during appendage regeneration. Typically in these vertebrates, amputated body parts are flawlessly repaired via the activation, proliferation, and patterning of progenitor cells (Tanaka, McCusker and Gardiner 2014). Wound-induced EFs likely affect the behavior of immune and progenitor cells because they are enacted during the
early wound-healing response, and interruption or reversal of an EF is
detrimental to regeneration (Borgens, Vanable et al. 1977, Borgens, Vanable et
al. 1979, Jenkins, Duerstock et al. 1996). However, exactly how cells detect and
translate EF information to elicit specific cell behaviors is not well understood.
One possibility is that information from an EF is modulated and transduced by ion
channels. Consistent with this idea, Ozkucur et al (Özkucur, Epperlein et al.
2010) showed that that ion concentrations are modulated in cells near the
amputation site during axolotl (*Ambystoma mexicanum*) tail regeneration. This
observation suggests that ion channels affect cellular behaviors by regulating ion
concentration-dependent signaling cascades during regeneration (Wondergem,

In this study, we performed a chemical genetic screen to identify ion channels
whose functions are required for normal axolotl tail regeneration. We
administered tail amputations to axolotl embryos and treated groups with ion
channel antagonists. Multiple channels were identified from several ion channel
families that either delayed the regenerative process or inhibited the initiation of
regeneration. Two of these channels, anoctamin 1 and voltage-gated K⁺
channels, were investigated further to determine their effects on cellular
proliferation and the activity of phagocytic cells.
3.3 Methods and Materials

3.3.1 Anesthesia

Experiments were conducted using pre-feeding, hatchling stage axolotl embryos (RRID: AGSC_100H, 9-11 mm in length) obtained from the Ambystoma Genetic Stock Center (RRID:SCR_006372), Department of Biology, University of Kentucky. Ethical animal procedures performed in this study were approved by the University of Kentucky IACUC committee (protocol 00907L2005). The skeletal muscle specific myosin inhibitor N-benzyl-p-toluene sulfonamide (BTS) was used to anesthetize embryos before all amputations and imaging procedures. Embryos were placed in modified Holtfreter’s solution (59mM NaCl, 0.67mM KCl, 0.76mM CaCl₂, 2.4mM NaHCO₃) with 10µM BTS at least 15 min before conducting any procedures.

3.3.2 Regeneration assay

Axolotl tails were imaged under a dissecting microscope and amputated approximately halfway between the tail tip and cloaca (~2mm). Embryos were then placed in individual wells of a 24-well tissue culture plate with 3 mL of Holtfreter’s solution containing various ion channel antagonists. The drug screen consisted of three tiers of ion channel antagonist ranging from broad scale general ion conductance inhibitors (tier 1), to antagonists of ion channel sub families (tier 2), and finally specific ion channel blockers (tier 3, table 3.1). All
drugs were initially evaluated at four concentrations (10^{-4}, 10^{-5}, 10^{-6} and 10^{-7}M) with 6 biological replicates for each concentration (some concentrations were lethal and this resulted in n<6 for some drug treatments). Subsequently, ranges of drug concentrations were evaluated on a case-by-case basis. Drugs that exhibited systemic toxicity (identified by general atrophy, lethargy and/or tissue degeneration) or lethality at all concentrations were excluded from the study. At 7 days post-amputation (dpa), embryos were anesthetized, imaged and tail length was measured. To assess the extent of regeneration, tail length at day 0 (Figure 3.1A) was subtracted from tail length at day 7 (Figure 3.1B). Results for each pharmacological agent were analyzed in Sigma Plot Statistical Software (SPSS) using a one-way ANOVA with Dunnett’s test for post hoc analysis. Significant results were qualitatively assigned as either delayed/reduced regeneration, inhibited regeneration or toxic/lethal (Figure 3.1B-E).

3.3.3 Phagocyte Imaging

Live imaging of phagocytes was accomplished by staining with the non-toxic dye, neutral red. Neutral red stains lysosomes which are present in many cell types but are especially rich in phagocytes such as macrophages and it has been confirmed as an effective and specific stain for macrophages in zebrafish and axolotl using the protocol described below (Davis and Ramakrishnan, Herbomel, Thisse et al. 2001, Godwin, Pinto et al. 2013). Prior to imaging, embryos were placed in Holtfreter’s solution containing 5 μg/mL of neutral red dye for 6 hours.
and then de-stained in normal Holtfreter’s solution for 24 hours. Following this staining/de-staining procedure embryos were anesthetized and imaged under bright-field (Herbomel, Thisse et al. 2001, Carradice and Lieschke 2008, Godwin, Pinto et al. 2013). Images were quantified by counting all labeled cells within 500 microns of the amputation plane and these values were normalized by tissue section area. Results were analyzed in SPSS using a one-way ANOVA with Dunnett’s test for post hoc analysis (control was t=0).

3.3.4 Proliferation assay

Axolotl embryos were amputated and placed in 24 well plates as outlined above. At 72 hpa, embryos were incubated for 16 hrs in 10 μM 5-ethynyl-2'-deoxyuridine (EdU), a BrdU analog. After this incubation period, embryos were euthanized and tails were amputated at the cloaca, immediately fixed in 4% paraformaldehyde and stored in 70% EtOH. Tissues were then embedded in paraffin wax and cut into 5-micron sagittal sections. The Click-iT® EdU Alexa Fluor® 488 Imaging Kit (C10337, invitrogen) was used to visualize EdU incorporation into the DNA of proliferating cells per the prescribed protocol. Hoechst was used to counter stain the sections. All cells staining positive for EdU incorporation within 500 microns of the amputation plane were counted as well as all cells staining positive for Hoechst within the same anatomical area. Proliferative index was determined by dividing the number of EdU+ cells by the number of Hoechst+ cells in each tissue section. Results were analyzed in
SPSS using either a one-way ANOVA with Dunnett’s test for post hoc analysis or, in the case of tissue specificity analysis, a two-way ANOVA (factors were tissue type and drug treatment) with Student-Newman-Keuls (SNK) test used for post hoc analysis.

3.3.5 Apoptosis assay

Axolotl embryos were administered tail amputations and placed in 24 well plates and allowed to regenerate for 72 hours. Embryos were euthanized and tails were amputated at the cloaca, immediately fixed in 4% paraformaldehyde and stored in 70% EtOH. Tissues were then embedded in paraffin wax and cut into 5-micron transverse sections. The Click-it® TUNEL Alexa Fluor® 594 Imaging Kit (C10246, invitrogen), was used to assay for fragmented DNA, a measure of apoptosis (Gavrieli, Sherman et al. 1992). Tissue sections were grouped as either 0-100 µm or 150-250 µm from the tail tip. TUNEL positive cells in each group were counted and divided by the total number of cells in each group (Hoechst) to give a measure of apoptotic index. Results were analyzed in SPSS using one-way ANOVA and SNK test for post hoc analysis.

3.3.6 RNA extraction and quantitative real-time PCR

Embryos were administered amputations and placed in 24-well plates as before and allowed to regenerate for 72 hours in either modified Holtfreter’s or modified
Holtfreter’s supplemented with the anoctamin 1 inhibitor, T16A(inh)-A01 (10 µM). The distal 1 mm of the tail was re-amputated and collected in ice-cold RNAlater (25 mM C₆H₇NaO₇, 10 mM EDTA, 5.3 M (NH₄)₂SO₄ and pH 5.2) and stored at -20°C. To isolate mRNA, 2 tails were placed in 100 µl of Trizol, homogenized via sonication and purified via Zymo Direct-zol™ RNA MiniPrep (R2050) kit and treated with DNase 1. RNA concentration and purity were determined via Nanodrop ND-1000 spectrophotometry (Nanodrop Technologies). cDNA was generated using qScript™ cDNA Supermix (Quanta Biosciences 95048) and used for PCR. PCR primers were designed using sequence data available on Sal-Site and are listed in table 3.2 (Baddar, Woodcock et al. 2015). Quantitative real-time PCR was performed using the StepOnePlus™ Real-Time PCR system (Applied Biosystems®). Samples were loaded into 96-well plates in triplicate with probes and Power SYBR® Green PCR Master Mix (4367659) to detect PCR products. Relative gene expression was calculated using the comparative \( \Delta \Delta C_T \) method \( (2^{-\Delta\Delta C_T}) \) in StepOne software v2.3 per the manufacturer’s instructions (Schmittgen and Livak 2008). Relative quantifications were compared in SPSS using a one-way ANOVA and Student–Newman–Keuls (SNK) method for post hoc analysis.
Table 3.1: This table summarizes all compounds and the concentrations of each compound screened for an effect on regeneration. Every concentration of each compound was screened using 6 or more animals. Concentrations were deemed to be lethal and/or systemically toxic (listed in bold) if 60% or greater of the animals in the screen either died or exhibited characteristics of toxicity (anatomical abnormalities in the gills, reduced blood flow in gills, general atrophy and degeneration).
Figure 3.1: Regeneration was assayed over the course of seven days. To quantitatively assess regeneration, two measurements were taken: (A) the first measurement was taken from the cloacal opening to the amputation plane immediately following amputation and (B) the second measurement was taken from the cloacal opening to the tip of the tail following 7 days of regeneration. Measurement A was subtracted from measurement B as a measure of regenerative growth. For qualitative analysis there were four possible outcomes for the assay. (1) Embryos exhibited a morphologically normal, regenerated tail, indicating the compound had no effect on regeneration. (2) Some regeneration occurred but the resulting tail was morphologically abnormal or not completely regenerated. (3) No regenerative outgrowth occurred beyond the amputation plane. (4) Embryos showed systemic toxicity, identified by general atrophy, lethargy and/or tissue degeneration (amputation plane indicated by black dashed line).
<table>
<thead>
<tr>
<th>GENE</th>
<th>FORWARD PRIMER</th>
<th>REVERSE PRIMER</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAPDH</td>
<td>ACGTCTCTGTGGTGGTACTTG</td>
<td>TCCCCTTCATTTGGGTCCATCAG</td>
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<tr>
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<td>CGCATGAAGCTATCCGACTT</td>
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<td>RAF1</td>
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<tr>
<td>MAPK3/ERK1</td>
<td>CGCAGTGGATCCTGCTGATAA</td>
<td>GGTTCGTAGTACTGTTCCAGGTA</td>
</tr>
</tbody>
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**Table 3.2:** This table summarizes the primers used to measure gene transcription by quantitative real-time PCR.
3.4 Results

3.4.1 Pharmacological Screen of Ion Channel Groups

3.4.1.1 Chloride

Nonspecific chloride conductance was probed with the broad-scale Cl⁻ channel blockers 4,4′-Diisothiocyanatostilbene-2,2′-disulfonic acid (DIDS), 4,4′-dinitrostilbene-2,2′-disulfonic acid (DNDS) and niflumic acid (NFA). Embryos treated with any of these broad scale Cl⁻ channel antagonists exhibited decreased tail regeneration at all concentrations tested, with the exception of DNDS at 1µM. For example, embryos exposed to 10μM DIDS, DNDS or NFA regenerated 0.71±0.31mm, 1.11±0.32mm and 0.63±0.24 mm respectively, significantly less than untreated embryos (Figure 3.2A). Calcium-activated Cl⁻ channels (CaCC) were evaluated with the family specific inhibitor CaCCinh-A01. CaCCinh-A01 was lethal at concentrations greater than 2µM but reduced regeneration at 0.1µM (1.01±0.24mm) and 1µM (1.09±0.17mm, Figure 3.2B) with no observable toxic effects. Anoctamins 1&2 (aka Tmem16A/B, Ano1, Ano2) were probed with benzbromarone (BBM) or T16a(inh)-A01 (A01). Embryos treated with either BBM or A01 had decreased regenerative capacity at 100 nM (0.56±0.18mm) and 10 µM (0.88±0.21mm, Figure 3.2C&D). The ligand gated Cl⁻ channels, GABAₐ receptors (GABAₐR) and glycine receptors (GlyR), were probed with picrotoxin (PTx) and bicuculline methiodide (BCU, GABAₐR
and both compounds caused reduced regenerative capacity at all concentrations tested. Both PTx (0.645±0.27mm) and BCU (0.618±0.32mm) elicited complete inhibition of regeneration at a concentration of 100µM (Figures 3.2E&F). All other Cl⁻ channel blockers were either toxic or did not affect regeneration (Figure 3.3).

3.4.1.2 Potassium

General potassium conductance was initially probed with the broad-scale K⁺ channel blocker tetraethylammonium (TEA). Compared to control embryos (1.83±0.38 mm), TEA treated embryos at a concentration of 1µM or higher decreased regenerative growth (0.96±0.29 mm, Figure 3.4A). The voltage-gated K⁺ channel (KV) blocker 4-aminopyridine (4-AP) reduced regenerative outgrowth at 4 µM (1.05±0.03 mm) and completely inhibited regeneration at 10 µM (0.50±0.24 mm) and 100 µM (0.59±0.27mm, Figure 3.2B). KV2.1/2.2 blockade with Guangxitoxin-1E (GTx-1E) slightly reduced regeneration at 1µM (1.11±0.46, Figure 3.4C). Kv1.5 blockade with cytochalasin B (CytB) had no effect on regeneration when embryos were incubated at 0.1 or 1µM, but tail outgrowth was inhibited at 10 µM (0.16±0.11 mm, Figure 3.4D). All other K⁺ channel blockers used were either toxic or did not affect regeneration (Figure 3.5).
3.4.1.3 Sodium

Sodium was assessed using only tier 2 antagonists as no tier 1 drugs were available. The epithelial sodium channel (ENaC) blocker amiloride did not affect regeneration at any concentration tested. The voltage-gated sodium channel (Nav) antagonists, tetrodotoxin (TTx) and lidocaine, also did not affect regeneration at any concentration tested, although, the highest concentration of TTx (100 μM) was lethal in 100% of embryos (Figure 3.6).

3.4.1.4 Calcium

Calcium conductance was probed with the broad-scale Ca\textsuperscript{2+} channel blocker bepridil (BPD). BPD was 100% lethal at every concentration tested. The L, N & T-type calcium channel inhibitor, benidipine HCl (BNP), completely inhibited regeneration at 100 nM (0.60±0.35mm) and all other concentrations were lethal. L-type channel blockers amlodipine (Ca\textsubscript{V}1.3) and diltiazem (Ca\textsubscript{V}1.1/1.2/1.4) diminished the regenerative response, with a maximal effects observed 10μM (1.03±0.38mm) and 1 μM (1.04±0.50mm), respectively. The P & Q-type blocker, ω-conotoxin MVIIC, completely inhibited regeneration with a maximal effect at 10 nM (0.57±0.19mm). P-type specific inhibitor, ω-agatoxin IVA, had no effect on the regenerative response at any concentration, thus eliminating Q-type channels as affecting regeneration (Figure 3.7).
3.4.1.5 Pumps/Transporters

The sarco/endoplasmic reticulum Ca\(^{2+}\)-ATPase (SERCA) was probed with 2,5-di-(tert-butyl)-1,4-benzohydroquinone (tBuBHQ); this chemical completely inhibited regeneration with maximal inhibition at 2 µM (0.41±0.15mm). Incubation with the H+/K+-ATPase inhibitor, pantoprazole, resulted in a slight reduction of regenerative growth at 100 µM (1.14±0.05mm). All other transporter inhibitors used were either toxic or did not affect regeneration (Figure 3.8).

3.4.2 Phagocyte activation is dependent on KV channel signaling

Macrophage activation is required for regeneration to proceed normally and KV channels are known to influence many functions of macrophages in vitro (Kotecha and Schlichter 1999, Qiu, Campbell et al. 2002, Vicente, Escalada et al. 2005, Godwin, Pinto et al. 2013, Moreno, Prieto et al. 2013). This suggests that KV’s may influence tail regeneration through modulation of the macrophage response. To test this hypothesis, it was first necessary to characterize the normal response of phagocytic cells during tail regeneration. Using neutral red (NR) to label phagocytic cells, a significant accumulation of phagocytes was observed at the wound site beginning 24 hours post amputation (hpa, 90.1±20.8 NR+ cells/mm\(^2\) tissue) and initially peaking at 48 hpa (189.2±53.7 NR+ cells/mm\(^2\) tissue). This was followed by a decline concurrent with tissue outgrowth and then a second peak at 144 hpa (200.6±34.4 NR+ cells/mm\(^2\) tissue, Figure 3.9).
NR stained cells were confirmed to be phagocytic by repeating the NR staining procedure on embryos that had been previously injected with liposome encapsulated clodronate. In comparison to control embryos, clodronate treated embryos exhibited a significant reduction of NR+ cells (Figure 3.10).

While CytB is a potent inhibitor of Kv1.5 channel activity, it has off target effects (such as disruption of actin polymerization) that could also influence regeneration. To further assess the role of Kv channels on phagocyte activity we used an alternate Kv channel blocker, 4-AP. Embryos were incubated at 2 concentrations of 4-AP (5 µM and 25 µM) beginning at 12 hpa and harvested at either 3 or 6 days post amputation (dpa). At 3 dpa, embryos incubated in either 5µM (45.88±12.35 NR+ cells) or 25 µM (46.63±10.07 NR+ cells) exhibited a marked reduction in phagocyte recruitment to the wound site compared to control embryos (123.33±15.67 NR+ cells) but there was no difference between the two concentrations tested. At 6 dpa, the reduction of phagocyte recruitment persisted compared to control (190.29±43.02 NR+ cells) and there was a concentration dependent response between the 5 µM group (102.38±9.89 NR+ cells) and 25 µM group (52.71±14.74 NR+ cells, Figure 3.11). To confirm this result, embryos were subjected to one of three treatments: (1) Encapsome®/Fluorosome®-DiI 50:50 mixture IP injection 24 hours before amputation (24hba), (2) Clodrosome®/Fluorosome®-DiI 50:50 mixture IP injection 24hba or (3) Encapsome®/Fluorosome®-DiI 50:50 mixture IP injection 24hba and incubation in 25 µM 4-AP beginning at 12 hpa. Embryos were then
harvested at 3 dpa and whole mount imaged under fluorescent and bright field conditions. Embryos subjected to both the phagocyte depletion protocol (17.045±17.30 Dil+ cells/mm²) and Kv channel inhibition (31.80±19.35 Dil+ cells/mm²) exhibited reduced phagocyte recruitment following amputation compared to control conditions (145.20±77.17 Dil+ cells/mm², Figure 3.12). Notably, the number of phagocytes at the amputation site at 3 dpa under control conditions identified by Fluorosome®-Dil incorporation was consistent with the number identified by NR staining at the same time point.

3.4.3 Ano1 blockade delays regeneration via inhibition of proliferation

Cell proliferative responses to amputation was assessed under several experimental interventions by measuring EdU incorporation at 72 hours post amputation (hpa). EdU incorporation was observed in 34.00±6.44% of cells within 500 microns of the amputation plane under control conditions (n=4). In contrast, broad-scale Cl⁻ channel blockade (DIDS: n=4, 18.13±5.52% & DNDS: n=5, 19.65±9.29%) or specific inhibition of anoctamin 1 (A01: n=5, 19.65±4.54%) significantly reduced the percentage of cells proliferating within this same anatomical area. Embryos incubated in an environment depleted of chloride (n=5) exhibited a trend towards reduced proliferation (24.80±9.74%) but the difference was not statistically significant relative to controls (Figure 3.13 A-F).

We note that apoptosis was assessed using a TUNEL assay; no differences
were observed between embryos subjected to anoctamin 1 blockade and controls (Figure 3.14).

To more finely examine proliferation spatially, cell counts were obtained for epidermal (ED), spinal cord (SC) and mesenchymal & muscle (MM) regions of the tail. Neither broad-scale Cl⁻ channel blockade (DIDS & DNDS) nor anoctamin 1 inhibition (A01) treatments affected proliferation rates within the ED and SC tissues; the reduced proliferation response was only observed in MM tissues. These results suggest that functional chloride flux mediated by Ano1 is critical for regulating the proliferative response in the mesenchyme but not in other tissues (Figure 3.13 G&H).

3.4.4 Ano1 blockade diminishes activation of p44/42 MAPK signaling pathway

To investigate gene expression changes associated with anoctamin 1 inhibition of tail regeneration, quantitative real-time PCR was used to measure mRNA expression of p44/42 MAPK signaling pathway genes, a pathway activated during cellular proliferation. After amputation, *pkcγ, mek1, mek2, erk1* and *erk2* showed a higher percent increase in transcription in control embryos compared to anoctamin 1 treated embryos at 72 hpa. In contrast, expression was downregulated more strongly in control embryos than embryos subjected to anoctamin 1 inhibition by A01 at 72 hpa for early p44/42 pathway genes (*pkca,*
pkcβ, ras and raft) (Figure 3.15). These results show that anoctamin 1 affects the transcription of p44/42 MAPK pathway genes that regulate cellular proliferation.
Figure 3.2: (A) All broad scale inhibitors of chloride channels (DNDS, DIDS and NFA) robustly reduced tail regeneration at concentrations of 100nM and above. (B-D) Inhibitors of calcium activated chloride currents with CaCCinh-A01, T16a(inh)-A01 and Benzbromarone all reduced regenerative growth in a concentration dependent manner with maximal responses at 2, 100 and 0.33 µM respectively. (E & F) PTx and BCU were used to investigate the role of ligand-gated chloride channels and both exhibited robust inhibition of regeneration at all concentrations tested (* indicates p<0.05 compare to control or concentration = 0; error bars are standard deviations).
Figure 3.3: Control means and standard deviations are depicted by the solid black and dashed grey lines (respectively) on each plot. (A) All broad scale (Tier 1) blockers of chloride channels resulted in delayed and/or reduced regeneration. (B) CaCCs are indicated by both the tier 2 blocker, CaCCinh-A01, and the tier 3 drugs Benz bromarone (Tmem16A/B) and T16Ainh-A01 (Tmem16A). (C) Ligand gated chloride channel blockers resulted in complete inhibition of regeneration at higher concentrations. (D) Blockade of volume regulated anion channels was lethal or caused systemically toxic at all concentrations tested. (E & F) Neither ClC of CFTR blockade had any effect on regeneration (* indicates p<0.05 compared to control measurements; error bars are standard deviations).
Figure 3.4: (A) While the broad scale potassium channel inhibitor TEA only reduced regenerative growth (B) the voltage-gated $K^+$ channel blocker 4-AP exhibited concentration dependent inhibition. (C) $K_{V2.1/2.2}$ channels that were probed with GTx-1E exhibited a slight reduction of regenerative growth at 1 and 5 $\mu$M but was lethal at all concentrations above 5 $\mu$M and (D) CB exhibited complete inhibition at 10 $\mu$M (* indicates p<0.05 compare to control or concentration = 0; error bars are standard deviations).
Figure 3.5: Control means and standard deviations are depicted by the solid black and dashed grey lines (respectively) on each plot. (A) Broad scale potassium channel blockade using Tetraethylammonium (TEA) reduced regenerative outgrowth (B). Tail regeneration was completely inhibited in a dose dependent manner by the KV Channel blocker 4-Aminopyridine (4-AP). (C & D) The more specific inhibitors of KV Channels were less effective at non-lethal concentrations with Cytochalasin B affecting regeneration the most (* indicates p<0.05 compared to control measurements).
Figure 3.6: Control means and standard deviations are depicted by the solid black and dashed grey lines on each plot. Broad scale sodium channel blockade was probed with either Amiloride, Lidocaine or Tetrodotoxin. None of these drugs affected regenerative growth, although tetrodotoxin was toxic at higher concentrations (* indicates p<0.05 compared to control measurements).
Figure 3.7: Control means and standard deviations are depicted by the solid black and dashed grey lines on each plot. Broad scale calcium channel blockade was probed with Bepridil HCl which was lethal or toxic at all concentrations tested. The more specific inhibitors of Cav Channels were more informative exhibiting reductions from either Amlodipine or Diltiazem (A). Complete inhibition was observed in animals treat with ω-conotoxin MVIIC but interestingly ω-agatoxin IVA had no effect. (* indicates p<0.05 compared to control measurements; error bars represent standard deviation)
Figure 3.8: Control means and standard deviations are depicted by the solid black and dashed grey lines on each plot. Ion pumps and transporters were probed only in tier 3 of the screen. H/K pump inhibitors resulted in reduced regeneration and Na/K pump inhibitors resulted in complete inhibition of regeneration. SERCA inhibitors inhibited regeneration but were systemically toxic to embryos. (* indicates p<0.05 compared to control measurements).
Figure 3.9: Phagocytes were stained in live regenerating axolotl embryos with neutral red at 6, 18, 24, 36, 48, 72, 120, 144 & 168 hpa. Panel (A) depicts representative micrographs of each time point (amputation plane indicated by black dashed line) with enlarged photos in panels (B & B’) showing individual phagocytes. (C) Phagocytes were counted from 500 µM proximal of the amputation plane to the tip of the tail and normalized by tissue cross-sectional area. Phagocyte density steadily increased over the first few days following amputation peaking between days 2 and 3. This was followed by a trough in phagocyte density between days 4 and 5 that corresponded to increased tissue outgrowth, and then a second phagocyte density peak at day 6 (* indicates p<0.05 compared to phagocyte density immediately following amputation; scale bars represent 1 mm; error bars are standard deviations).
Figure 3.10: To confirm NR staining phagocytes, embryos were injected IP with either Encapsome® for vehicle control or Clodrosome® for macrophage depletion. Macrophage depletion (A&C, n=17) significantly reduced the number of NR+ cells at the wound site following amputation compared to control embryos (A&B, n=15). (* indicates p<0.05 compared to control measurements; error bars are standard deviations; amputation plane indicated by black dashed line).
Figure 3.11: Phagocytes were stained in live animals with neutral red at 3 and 6 dpa. Panel (A) depicts representative micrographs of all time points and treatments (amputation plane indicated by black dashed line). (B) 4AP inhibits phagocyte activation and recruitment to the wound site at concentrations of either 5 or 25µM. This response is concentration dependent at 6dpa but not 3dpa (* indicates p<0.05 compared to control and ^ indicates p<0.05 compared animals treated with 5µM 4AP; scale bars represent 1 mm; error bars are standard deviations).
Figure 3.12: Phagocytes were labeled by IP injection of Fluorosome®-Dil 24 hours prior to amputation and co-injected with either Encapsome® as a vehicle control (B-B”; n=10), Clodrosome® for macrophage depletion (C-C", n=15) and a final group co-injected with Encapsome® and then incubated with 25µM 4-AP following amputation for Kv channel blockade (D-D”; n=14). (* indicates p<0.05 compared to control; error bars are standard deviations) Both macrophage depletion prior to amputation (C-C") and Kv channel blockade following amputation (D-D") reduced the number of Dil+ cells at the wound site (amputation plane indicated by white dashed line).
Figure 3.13: Compared to controls (n=4) (A-A’), the proliferative response to amputation was reduced by broad scale chloride channel blockade with either DIDS (n=4) or DNDS (n=5) (C) as well as anoctamin-1 channel blockade with T16a(inh)-A01 (n=5) (B-B’)) but not when animals were incubated in chloride free medium (n=5) (C) (amputation plane indicated by white dashed line). Taken together these data suggest that chloride channel signaling is a critical step in the mechanism(s) driving proliferation in response to amputation. (* indicates p<0.05 compared to control; error bars are standard deviations) (D&E) Neither DIDS nor T16a(inh)-A01 had an effect on proliferation in spinal cord (SC) or epidermal (ED) tissues. In mesenchymal tissues (MM) directly underlying the wound epidermis and within 500 microns of the amputation plane, proliferation was reduced from 46.6±0.7% in control animals to either 20.0±3.5% (DIDS) or 23.0±2.9% (T16a(inh)-A01) in treated animals. (* indicates p<0.05 compared to control of same tissue type; error bars are standard deviations).
Figure 3.14: Apoptosis was assessed following amputation in 3 dpa animals and compared to apoptotic rates in tissue from un-amputated axolotl tails. Tissues were cut into transverse sections and then divided into groups consisting of sections of either from the tail tip to 100 µM distal of the amputation plane and a second group consisting of tissue from 150 to 250 µM distal of the amputation plane. No differences were observed in the latter group but the former exhibited similar increases in apoptotic cells in both the control 3dpa animals and 3dpa animals treated with the Ano1 inhibitor. (* indicates p<0.05 compared to un-amputated tail tissue).
Figure 3.15: Quantitative Real-Time PCR was used to assess the mRNA expression of genes associated with the p44/42 MAPK (Erk 1/2) signaling pathway at 72 hours post amputation (hpa) in regenerating axolotl tails and at the same time point in animals treated with 20µM T16Ainh-A01. Expression in tail tissues from un-amputated animals was used as control/baseline. All genes other than PKCγ exhibit modulated expression at 3dpa, which was attenuated by Ano1 blockade (* indicates p<0.05 compared to control and ^ indicates p<0.05 compared to normal 72 hpa animals; error bars are standard deviations).
3.5 Discussion

In this study, we identified ion channel antagonists that partially (anoctamin1/Tmem16a, anoctamin2/Tmem16b, Kv2.1, Kv2.2, L-type Cav channels and H/K ATPases) or completely (GlyR, GABA_{\text{A}}R, Kv1.5 and SERCA pumps) inhibited axolotl tail regeneration. Below we separately discuss the affected ion channels from each of these categories (Figure 3.16).

3.5.1 Partially Inhibiting Channels & Transporters

3.5.1.1 Anoctamins 1/2 (Tmem16A/B)

Our results suggest a role for CaCCs in the tail regeneration process. Regeneration was delayed when embryos were subjected to either broad-scale Cl\(^{-}\) channel blockade (DIDS, DNDS & niflumic acid) or CaCC inhibition (CaCCinh-A01, T16a(inh)-A01 & Benzbromarone). CaCC currents were described over 25 years ago but their molecular basis was only discovered recently (Hartzell, Putzier et al. 2005). In 2008, three independent researchers identified anoctamin 1 and anoctamin 2 channels as mediators of CaCC currents (Caputo, Caci et al. 2008, Schroeder, Cheng et al. 2008, Yang, Cho et al. 2008). Anoctamin 1 was first discovered as an overexpression marker for gastrointestinal stromal tumors (then known as DOG1) and has since been shown to promote other forms of cancer (West, Corless et al. 2004, Britschgi, Bill et al. 2013). Blockade of anoctamin 1 suppresses tumor growth and invasion in multiple human cancer
There is evidence that anoctamin 1 manifested these effects through regulation of both cell migration and proliferation (Ruiz, Martins et al. 2012, Qu, Yao et al. 2014). Our results support this idea because inhibition of anoctamin 1 significantly decreased the number of proliferating cells in the regenerating tail. This suggests that anoctamin 1 channel function is directly or indirectly required to sustain cell proliferation at a level that is typical of normal tail regeneration.

Our results further suggest that anoctamin 1 affected tail regeneration by modulating the p44/42 MAPK pathway, which is a well-known regulator of cell proliferation (Zhang and Liu 2002). Following tail amputation, Erk1/2 signaling pathway genes were transcriptionally upregulated to a higher degree in control embryos than embryos treated with anoctamin 1 antagonist. Thus, the magnitude of erk1/erk2 transcription correlated on one hand with anoctamin 1 function, and conversely with the magnitude of cell proliferation. We propose the following model: anoctamin 1, activated by an initial calcium surge, acts as a countercurrent ion channel to amplify calcium signaling. This amplified surge subsequently activates the Erk1/2 pathway leading to increased cellular proliferation. This model assumes there is a burst in intracellular [Ca^{2+}] following tail amputation and a dependence of intracellular Ca^{2+} flux on chloride conductance. Ozkucur et al (Özkucur, Epperlein et al. 2010) downplayed the role of Ca^{2+} in their model of axolotl tail regeneration. However, they reported significant increases in Ca^{2+} fluorescence at 48 hpa, preceding the time when the...
tail shows measureable regenerative outgrowth. This Ca\(^{2+}\) flux at 48 hpa supports our proposed model (Wang and van Breemen 1999, Barro-Soria, Aldehni et al. 2010, Özkucur, Epperlein et al. 2010).

3.5.1.2 Kv2.1/2.2

Kv2 channel blockade with TEA or GTx-1E also reduced regeneration. Kv2 channels traditionally act as the primary delayed rectifier involved in regulating the excitability of neurons and facilitating exocytosis in neurons and neuroendocrine cells (Misonou, Mohapatra et al. 2005, Feinshreiber, Singer-Lahat et al. 2009). These functions of Kv2 channels could be associated with the release of neurotrophic factors that are required for regeneration, the so-called neurotrophic requirement for regeneration (Singer 1974, Kumar and Brockes 2012). However, it is also important to consider the direct influence of Kv2 channels on the cellular dynamics of non-excitible cells (Kumar and Brockes 2012). Kv2 acts as a promoter of cell migration in cultured HEK293, CHO and bone derived mesenchymal stem cells (MSC) via phosphorylation of focal adhesion kinase (Wei, Wei et al. 2008, Hu, Wei et al. 2011). MSC also require functioning Kv2 channels for proper progression through the cell cycle by facilitating plasma membrane-ER contact sites (Deng, Lau et al. 2007, Cobb, Austin et al. 2015). These MSC specific characteristics of Kv2 channels are particularly interesting considering their resemblance to progenitor cells of the
blastema, but more research is required to determine the specific role of Kv2 channels within the context of appendage regeneration.

3.5.1.3 L-type Ca\textsuperscript{2+} channels (Ca\textsubscript{v1.1-1.4})

The L-type channel blockers amlodipine and diltiazem partially inhibited tail regeneration. These channels are mostly known for their roles in excitable cells, where they couple plasma membrane depolarization with increases in Ca\textsuperscript{2+} conductance across the membrane. Recent evidence also suggests that they are important physiological components of many non-excitable cell types (Wen, Wang et al. 2012, Davenport, Li et al. 2015). Ca\textsuperscript{2+} is a ubiquitous second messenger in all cell types and can influence many different cellular processes (Berridge 1995, Berridge, Lipp et al. 2000, Zayzafoon 2006, Clapham 2007, Zheng and Poo 2007). Because of the widespread nature of L-type Ca\textsuperscript{2+} channel expression and the complicated features of intracellular Ca\textsuperscript{2+} signaling, these channels may not present the best targets for future investigations. But, if the model we proposed above is correct, the anoctamin 1/2 Cl\textsuperscript{-} channels may provide an indirect path to modulate intracellular calcium flux in a specific cell population.
3.5.1.4 H+/K+ ATPases

Our study found that H+ pump inhibition with pantoprazole sodium reduced regenerative outgrowth. Proton pumps have been classically associated with their roles in gastric and renal function, however over the past several decades they have been identified as critical mediators of wound healing and regeneration (Walan, Bader et al. 1989, Adams, Masi et al. 2007). Most of this influence has been attributed to the proton pump’s involvement in establishing and maintaining trans-epithelial electrical potentials that drive wound-induced electrical fields (Nuccitelli 2003). A recent chemical genetic screen performed using planarian head regeneration also identified H+/K+ ATPase as important for initiating the regenerative process through control of cellular membrane potentials ($V_{mem}$) (Beane, Morokuma et al. 2011). H+ pump driven changes in $V_{mem}$ are required for regeneration to proceed normally in the Xenopus model of regeneration as well (Adams, Masi et al. 2007). Our findings further validate the requirement of H+ pumps in organisms capable of appendage regeneration.

3.5.2 Inhibiting Channels & Transporters

3.5.2.1 Kv1.5

Blockade of Kv1.5 with 4-AP or CytB resulted in a robust and concentration dependent total inhibition of regeneration. These channels are widely expressed
in a large number of tissues and are involved in regulating $V_{\text{mem}}$ and
electrophysiological properties of a variety of cell types (Archer, Souil et al. 1998,
Olson, Alekseev et al. 2006, Tabarean 2014). In addition to these
electrophysiological functions, $K_V1.5$ channels have been implicated in regulating
cell cycle progression and migration (Kotecha and Schlichter 1999, Villalonga,
interest is their role in regulating immune cell dynamics. Macrophages are
required for amphibian regeneration and the invertebrate homologue of
macrophages (hemocytes) respond to exogenously applied electrical fields in
vivo in a $K_V$ dependent manner (Godwin, Pinto et al. 2013, Franklin, Maroudas et
al. 2016, Li, Levin et al. 2016). Consistent with this line of reasoning, phagocyte
activation was severely diminished during tail regeneration after $K_V$ channel
blockade with 4-AP. This suggests that $K_V$ channels may be critical regulators of
macrophage/monocyte populations during regeneration however, further studies
are necessary to further clarify this role for $K_V$ channels in regeneration.

3.5.2.2 GlyR/GABA$_A$R

GlyR and GABA$_A$R blockade with picrotoxin or bicuculline resulted in strong
inhibition of regeneration at relatively low concentrations. These ligand-gated
chloride channels act primarily as receptors for inhibitory neurotransmitters in the
CNS with GABA$_A$R acting mostly in the brain and GlyR functioning primarily in
the brainstem and spinal cord (Lynch 2004, Sigel and Steinmann 2012). This
immediately implicates nerves and their required neurotrophic factors for appendage regeneration since modulation of either of these receptors would disrupt normal neuronal activity during regeneration. There is evidence that these receptors influence neural progenitor cells during early development and regulate many critical cellular processes in multiple cell types, mostly via modulation of intracellular Ca$^{2+}$ concentrations (Nguyen, Rigo et al. 2001, Van Den Eynden, SahebAli et al. 2009). More research will be necessary to assess the exact role of GlyR/GABA$_{\alpha}$R during axolotl regeneration.

3.5.2.3 SERCA pump

SERCA inhibition with tBuBHQ completely inhibited regeneration at low concentrations, although higher concentrations of this drug were lethal. The sacro/endoplasmic reticulum Ca$^{2+}$ ATPase (SERCA) is expressed in virtually all cell types and distributes Ca$^{2+}$ ions against their concentration gradient into sarcoplasmic and endoplasmic reticula. This allows for quick sequestration of cytoplasmic free Ca$^{2+}$ following a Ca$^{2+}$ signaling event and for the generation of intracellular calcium stores that allow for a more rapid and robust Ca$^{2+}$ surge upon cellular stimulation (Wuytack, Raeymaekers et al. 2002). As discussed above, Ca$^{2+}$ signaling is associated with many cytosolic signaling pathways and physiological functions, all of which require precise control of intracellular Ca$^{2+}$ concentration pulses in terms of both magnitude and duration, and these Ca$^{2+}$ dependent pathways are operative in models of epimorphic regeneration.
(Berridge, Lipp et al. 2000, Rao, Song et al. 2014). The ubiquitous nature of SERCA expression may explain why inhibition of this ion channel completely inhibited regeneration, while incomplete regeneration was observed for L-type Ca\textsubscript{v} channel inhibition. Based on the extensive network of pathways influenced by Ca\textsuperscript{2+} signaling and the pervasive nature of SERCA pumps, SERCA pumps will present challenging targets to investigate mechanisms of tissue regeneration.
Figure 3.16: A comprehensive screen of ion channel blockers was used to identify specific channels involved in critical cellular processes of growth and regeneration such as proliferation, migration and differentiation. Drugs that did not affect regeneration are depicted in green, drugs that resulted in partial regeneration are depicted in yellow, drugs that completely inhibited regeneration (for any of the concentrations tested) are depicted in red and drugs that were lethal at all concentrations or caused systemic toxicity (identified by general atrophy, lethargy and/or tissue degeneration) are depicted in black.
3.6 Summary

A chemical genetic screen was performed to identify ion channel antagonists that inhibit axolotl tail regeneration. Then, experiments were performed to determine how select antagonists affected cellular behaviors (cell proliferation and phagocyte activation) that are required for successful tail regeneration. The antagonists that were identified from the screen targeted the following ion channels: anoctamins 1/2 (Tmem16a/b), GlyR, GABAₐR, Kv1.5, Kv2.1, Kv2.2, L-type Cav channels, H/K ATPases and SERCA pumps. An association was established between Kv channel blockade and phagocyte activation, and thus a possible mechanism for Kv channel mediated inhibition of axolotl tail regeneration. Also, blockade of the anoctamins reduced cellular proliferation, and this was associated with modulated transcription of Erk1/2 signaling pathway genes. The antagonists and ion channels prioritized from our study will provide useful tools and targets for investigating mechanisms of tissue regeneration.
3.7 Acknowledgements

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CHAPTER 4
THE CALCIUM-ACTIVATED CHLORIDE CHANNEL, ANOCTAMIN-1,
REGULATES CELLULAR PROLIFERATION VIA ERK1/2
PHOSPHORYLATION AND CALCIUM SIGNALING

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Abbreviations used: Ano1 (anoctamin 1), Ca²⁺ (calcium cation), mANO1 (mouse anoctamin 1), HEK293 (human embryonic kidney cell line 293), CRISPR (clustered regularly interspaced short palindromic repeats), Cas9 (CRISPR associated protein 9), ATP (adenosine triphosphate), Erk (extracellular signal regulated kinase), Cl⁻ (chloride anion), PKC (protein kinase C), CO₂ (carbon dioxide), EDTA (Ethylenediaminetetraacetic acid), DMEM (Dulbecco’s Modified Eagle Medium), SF/AF-DMEM (serum-free, antibiotic-free DMEM), DNA (deoxyribonucleic acid), μL (microliter), RNA (ribonucleic acid), mRNA (messenger RNA), GFP (green fluorescent protein), PBS (phosphate buffered saline), cDNA (complementary DNA), PCR (polymerase chain reaction), qPCR (quantitative PCR), GAPDH (Glycerol-3-phosphate dehydrogenase), hANO1 (human anoctamin1), DMSO (dimethyl sulfoxide), μM (micromolar), NaCl (sodium chloride), KCl (potassium chloride), CaCl₂ (calcium dichloride), MgCl₂ (magnesium dichloride), Na₂HPO₄ (sodium phosphate dibasic), KH₂PO₄ (potassium dihydrogen phosphate), NaC₆H₁₁O₇ (sodium gluconate), K₃H₁₁O₇ (potassium gluconate), Ca(C₆H₁₁O₇)₂ (calcium gluconate), Mg(C₆H₁₁O₇)₂ (magnesium gluconate), FITC (Fluorescein isothiocyanate), mM (millimolar), MQAE (N-(Ethoxycarbonylmethyl)-6-Methoxyquinolinium Bromide), DAPI (4',6-Diamidino-2-Phenylindole, Dihydrochloride), PCNA (proliferating cell nuclear antigen), μg (microgram), PVC (polyvinyl chloride), pH (potential of hydrogen), BSA (bovine serum albumin), TBST (tris buffers saline with tween), mg (milligram), mL (milliliter), DSHB (developmental studies hybridoma bank), HRP (horseradish peroxidase), H₂SO₄ (sulfuric acid), nm (nanometer), PVDF (polyvinylidene difluoride), kDa (kilodalton), IgG (immunoglobulin G), ECL (enhanced chemiluminescence), S.E.M. (standard error measurement), SD (standard deviation), ANOVA (analysis of variance), SNK (Student-Newman-Keuls), [Ca²⁺]ᵢ (intracellular Ca²⁺ concentrations), RQ (relative quantity), A.U. (arbitrary unit), sec (second), SS (serum starved), CAMKII (calmodulin-dependent protein kinase II), Akt (protein kinase B), IP₃ (inositol 1,4,5-trisphosphate), PLC (phospholipase C), PMCA (Plasma membrane calcium ATPase), SERCA (sarcoplasmic/endoplasmic reticulum calcium ATPase), RKIP (Raf Kinase Inhibitory Protein), Raf-1 (Raf-1 Proto-Oncogene, Serine/Threonine Kinase), Pyk2 (protein tyrosine kinase 2-beta), SHR (spontaneously hypertensive rats), VSMC (vascular smooth muscle cells), WKY (Wistar-Kyoto rat), CaCC (calcium activated chloride channel)
Keywords: Calcium signaling, anoctamin 1, counter-current ion channel, calcium activated chloride channels, proliferation, HEK293, PKC, Erk1/2

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4.1 Abstract

Recent data indicate the calcium activated chloride channel anoctamin 1 (Ano1) as a regulator of cellular proliferation in several biological context including both normal physiology and pathophysiology. However the specific physiological mechanisms involved in manifesting this influence have remained elusive. Intracellular free Ca\(^{2+}\) is a well-known regulator of cell survival and growth pathways and provides a convenient second messenger system that may be sensitive to Ano1’s function as a chloride channel. Surges in intracellular Ca\(^{2+}\) concentrations are sensitive to Ano1 function indicated by amplification of Ca\(^{2+}\) surges in response to transient overexpression of mANO1 in HEK293 cells and attenuation of Ca\(^{2+}\) surges in response to both pharmacological inhibition of Ano1 and CRISPR/Cas9 mediated knock out of ANO1 in HEK293 cells. This Ano1 mediated amplification of Ca\(^{2+}\) surges is sufficient to drive cellular proliferation indicated by increased expression of proliferating cell nuclear antigen in response to ATP stimulation of serum starved HEK293 cells but not HEK293\(^{\text{ANO1-KO}}\) cells. We also show an Ano1 dependent activation of Erk1/2 signaling that is sufficient to initiate cellular proliferation. These data suggest a novel physiological regulator of intracellular calcium handling that regulates the versatile Erk1/2 signaling cascade which may have far reaching implications for human health and disease.
4.2 Introduction

Precise regulation of cellular growth and proliferation is a vital function in virtually all biological systems. Normal and constant cell turnover in skin, blood, and gastrointestinal tissues are critical for maintaining physiological function as well as stimulation and direction of proliferation in response to soft tissue injury. These processes therefore, facilitate wound-healing, tissue regeneration and repair. Disruptions to the normal regulation of this critical cellular process can lead to significant tissue pathologies. The biochemical regulators and checkpoints that control the cell cycle have been extensively investigated (Duronio and Xiong 2013). Ion-channel signaling has now been identified as a key contributor to the complex regulation of tissue homeostasis and its role in regulation of proliferation (Blackiston, McLaughlin et al. 2009).

Anoctamin 1 (Ano1) was recently reported as the molecular determinant of the calcium activated chloride current (Caputo, Caci et al. 2008, Schroeder, Cheng et al. 2008, Yang, Cho et al. 2008). Since this discovery, Ano1 has been functionally implicated as a regulator of proliferation in both normal physiologic context and the pathophysiologic generation of many cancers (Stanich, Gibbons et al. 2011, Qu, Yao et al. 2014). ANO1 overexpression has been identified in several cancer types where it impacts cellular proliferation and migration and is correlated with poor prognosis (Qu, Yao et al. 2014). Ano1 was recently
identified in a chemical genetic screen of amphibian appendage regeneration performed in our lab and this association was shown to be a consequence of Ano1’s regulation of cellular proliferation. A common theme that persist in the literature concerning Ano1’s influence on proliferation is that the Erk1/2 signaling pathway is involved (Duvvuri, Shiwarski et al. 2012, Britschgi, Bill et al. 2013, Deng, Yang et al. 2016). Despite these encouraging findings, the molecular mediators of Ano1’s influence on Erk1/2 signaling remain unknown.

In the current study, we investigated the molecular mediators of Ano1’s influence on Erk1/2 signaling and proliferation using genetic modification and pharmacological inhibition of Ano1 function. It was found that Ano1 amplifies intracellular Ca\(^{2+}\) surges in HEK293 cells and that this is correlated with an increase in intracellular Cl\(^{-}\) concentration. Furthermore, we show that this intracellular Ca\(^{2+}\) surge is sufficient to increase PKC activity, Erk1/2 signaling and cellular proliferation all in an Ano1 dependent manner.
4.3 Methods and Materials

4.3.1 Cell culture

Human Embryonic Kidney (HEK) 293 Cells were acquired from American Type Culture Collection (CRL-1573; ATCC, Manassas, CA) and expanded at 37°C in 5% CO₂. Cells were grown in Dulbecco's Modified Eagle's medium (DMEM; Sigma® D5523) supplemented with 10% fetal bovine serum (Gibco™: 10437010) and 1% Penicillin/Streptomycin cocktail (Gibco™: 15140122). Media was changed every other day and cells were passaged at 90% confluence with Trypsin-EDTA (Corning®: 25053Cl). HEK293ANO1-KO and HEK293mANO1 cells were cultured under identical conditions.

4.3.2 Cell transfection

The day prior to transfection cells were trypsinized and $6.0 \times 10^5$ were seeded in each well of 6-well culture plates to achieve ~70% confluence and incubated in fresh medium overnight. One hour prior to transfection medium was replaced with serum-free, antibiotic-free DMEM (SF/AF-DMEM). For each well to be transfected 3µg of DNA and 6 µL of P3000™ reagent was added to 150µL of SF/AF-DMEM and 4µL Lipofectamine™ 3000 was added to a separate tube of 150µL of SF/AF-DMEM. The diluted DNA and P3000 solution was added to the diluted Lipofectamine™ 3000 were combined and incubated at room temperature
for 20 minutes. The transfection solution was added drop wise to each well.

Following a 16-hour incubation at 37°C, SF/AF-DMEM was replaced with regular growth medium and cells were analyzed for expression the next day.

4.3.3 Transient overexpression of mANO1

A mouse anoctamin-1 (mANO1) expression plasmid (OriGene Technologies, Inc.: MC205263) was transfected into HEK293 cells by the protocol described above. Following 24-hour incubation in normal growth medium, transfected cells were either prepared for experiments or total mRNA was extracted for expression analysis.

4.3.4 CRISPR/Cas9 mediated Knockout of endogenous Ano1

A CRISPR/Cas9 knockout plasmid cocktail containing three ANO1 specific guide RNAs (Santa Cruz Biotech.: sc-401314) was transfected into HEK293 cells as described above. Following 24-hour incubation in normal growth medium cells were visually assessed for transfection efficacy via GFP expression and transfection efficiencies were routinely 80-90%. Cells we expanded through two passages and then either cryopreserved for future use, prepared for experiments or total mRNA was extracted for expression analysis.
4.3.5 RNA extraction and real-time quantitative reverse transcription polymerase chain reaction

To isolate mRNA, cells in 6-well plates were first washed in ice cold PBS and then lysed with 750μL of Trizol. mRNA was purified from lysates via Zymo Direct-zol™ RNA MiniPrep (R2050) kit and treated with DNase 1. RNA concentration and purity were determined via Nanodrop ND-1000 spectrophotometry (Nanodrop Technologies). cDNA was generated using qScript™ cDNA Supermix (Quanta Biosciences 95048) and used qPCR. Pre-designed TaqMan® gene expression primers and hydrolysis probes for all genes of interest were purchased (Applied Biosystems®: mANO1 - Mm00724407_m1; hANO1 - Hs00216121_m1; hGAPDH - Hs03929097_g1). mANO1 and hANO1 primer probe kits were mixed 50:50 in order to measure total ANO1 transcript levels and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression was used to normalize all samples. Quantitative real-time PCR was performed using the StepOnePlus™ Real-Time qPCR system (Applied Biosystems®). Samples were loaded into 96-well plates in triplicate with probes and Power TaqMan® Fast Advanced PCR Master Mix (4444557) to detect PCR products. Relative gene expression was calculated using the comparative C_{T} method (2^{-\Delta\Delta CT}) in StepOne software v2.3 per the manufacturer’s instructions (Schmittgen and Livak 2008).
4.3.6 Calcium Imaging

The day prior to calcium imaging cells were trypsinized and seeded near confluence in 96-well plates and incubated overnight in normal growth medium. Fluo-4 Am (50µg; Molecular Probes™: F14201) was reconstituted in 50µL of 20% Pluronic® F-127 in DMSO (Molecular Probes™: P3000MP). Fluo-4/Pluronic® F127 solution is diluted in DMEM for a final fluo-4 concentration of 10µM and cells are incubated in this solution at room temperature for 45 minutes in the dark. Cells are then washed with PBS 2-3 times and incubated in either physiological PBS (137 mM NaCl, 2.7 mM KCl, 1 mM CaCl₂, 0.5 mM MgCl₂, 10 mM Na₂HPO₄ and 1.8 mM KH₂PO₄) or modified Cl⁻ Free PBS (137 mM NaC₆H₁₁O₇, 2.7 mM KC₆H₁₁O₇, 1 mM Ca(C₆H₁₁O₇)₂, 0.5 mM Mg(C₆H₁₁O₇)₂, 10 mM Na₂HPO₄ and 1.8 mM KH₂PO₄) for calcium imaging. Cells were allowed to equilibrate to these solutions for five minutes and then transferred to an inverted microscope. Cells were imaged under a FITC filter every 5 seconds for 250 seconds. Cells were pulsed with 50 µM ATP to stimulate calcium entry at ~12.5 seconds after the first image was taken.

4.3.7 Chloride Imaging

The day prior to chloride imaging cells were trypsinized and seeded near confluence in 96-well plates and incubated overnight in normal growth medium. The following day cells were incubated in medium containing 5mM N-(Ethoxycarbonylmethyl)-6-Methoxyquinolinium Bromide (MQAE, Enzo Life
Sciences: 52156) for 2 hours at 37ºC. Cells are then washed with PBS 2-3 times and incubated physiological PBS for chloride imaging. Cells were allowed to equilibrate to this solution for five minutes and then transferred an inverted microscope. Cells were imaged under a DAPI filter every 5 seconds for 160 seconds. Cells were pulsed with 50 µM ATP to stimulate calcium entry at ~12.5 seconds after the first image was taken.

4.3.8 Enzyme-Linked Immunosorbent Assay

Polyclonal capture PCNA antibody (Novus Biologicals, LLC, Littleton, CO: NBP2-26154) was diluted 1:500 (1µg/mL) in carbonate/bicarbonate buffer (pH 9.6) and 100 µL was added to each well of a PVC 96-well micro titer plate. The plate was covered with adhesive film and incubated overnight at 4ºC. The next day capture antibodies were poured off and wells were washed 3X with PBS. 250 µL of blocking buffer (5% BSA in TBST) was added to each well and incubated overnight at 4ºC. Blocking buffer was poured off. Cell lysates were diluted to 2mg/mL in 0.15 mM NaCl and 100 µL were added to wells in duplicate. The plate was covered with an adhesive film and incubated for 2 hours at 37 ºC. Samples were poured off and wells were washed 3X with PBS. Monoclonal detection antibody (DSHB, Iowa City, IA: CPTC-PCNA-1) was diluted 1:400 in blocking buffer and 100 µL was added to each well and incubated 2 hours at room temperature. Detection antibodies were poured off and wells were washed 3X with PBS. Anti-rabbit IgG, HRP-linked secondary antibody (Cell Signaling
Technology® (CST), Danvers, MA: 7074) was diluted 1:1000 in blocking buffer and 100μL were added to each well and incubated 2 hours at room temperature. Secondary antibodies were poured off and wells were washed 3X with PBS. TMB Plus (100μL, Amresco, Solon, OH: K830) was added to each well and incubated at room temperature for 10 min. Stop solution (100μL of 2M H₂SO₄) was added to each well and absorbance at 450 nm was measured by a plate reader.

4.3.9 Western Blot

Protein samples were electrophoretically separated on 10% Mini-PROTEAN® TGX™ gels (Bio-Rad Laboratories, Inc., Hercules, California: 4561033) at 150V for 1 hour. Separated proteins were transferred by electroelution (40V, 1-2hrs) to polyvinylidene difluoride (PVDF) membranes (0.45μm; Millipore; Bedford, MA). Molecular weight markers 9-200kDa; Biotinylated Protein ladder (CST, Danvers, MA: 7727) were used to estimate molecular mass. Blots were blocked with 5% bovine serum albumin (BSA) in Tris-buffered saline with 20% tween (TBST). Blots were incubated with primary antibodies phospho-(Ser) PKC substrate antibody (1:750; CST, Danvers, MA: 2261), phospho-MSK1 (Thr581) (1:750; CST, Danvers, MA: 9595), phospho-p44/42 MAPK (Erk1/2) (1:1000; CST, Danvers, MA: 4370) and p44/42 MAPK (Erk1/2) (1:750; CST, Danvers, MA: 4695) at 25°C for 2 hours. Blots were washed with Tris-buffered saline/0.1% Tween-20 (TBST) and Blots were incubated with HRP conjugated secondary
antibodies anti-rabbit IgG (1:1000; CST, Danvers, MA: 7074) and anti-biotin (1:1000; CST, Danvers, MA: 7075) at 25°C for 2 hours. Detection of specific proteins was accomplished using Amersham ECL Prime Western Blotting Detection Reagent (GE Life Sciences, Pittsburgh, PA: RPN2232) according to manufacturer’s instructions, and imaged with a UVP ChemiDoc-It® Imager. Densitometric results were reported as integrated values (area density of band) and expressed as a ratio of target to loading control (ERK1/2). Results were then compared between treatment groups. Densitometry reflects mean ± S.E.M. of all samples.

4.3.10 Statistical Analysis

Data are expressed as mean ± SD. Comparisons between experimental groups were evaluated using one-way ANOVA and Student-Newman-Keuls (SNK) post-hoc analysis. Statistically significance was determined using a p< 0.05 threshold.
4.4 RESULTS

4.4.1 ATP stimulation of Ca$^{2+}$ surges in HEK293 cells is attenuated with anoctamin-1 blockade

Intracellular Ca$^{2+}$ concentrations ([Ca$^{2+}$]) of HEK293 cells in physiological PBS was monitored by fluo-4 intensity. Upon stimulation with 50 μM ATP these cells exhibited an increase in [Ca$^{2+}$], achieving a 4-fold increase in fluo-4 intensity in 42.27±10.09 seconds. Fluo-4 intensity stabilized 2.5 minutes following stimulation but maintained a 2-fold increase compared to baseline levels (Figure 4.1). Genetic modulation of Ano1 expression was achieved by either transient overexpression of mANO1 or Crispr/Cas9 mediated knockout for ANO1 (Figure 4.2). Anoctamin-1 blockade with T16a(inh)-A01 (20 μM) results in a reduction of this [Ca$^{2+}$] surge following ATP stimulation but has no effect on the velocity of Ca$^{2+}$ entry (Figure 4.3 B, B’, E & F). Maintaining these cells in modified Cl$^{-}$ free PBS completely abolishes the initial Ca$^{2+}$ response to ATP, although there is a gradual rise in [Ca$^{2+}$] resulting in a similar intensification of fluo-4 signal compared to the end of the control experiment. These data suggest that Ca$^{2+}$ surges are amplified by Ano1 dependent Cl$^{-}$ flux across the cell membrane.
4.4.2 Ca\textsuperscript{2+} surges stimulate Cl\textsuperscript{-} entry in HEK293 cells in an Ano1 dependent manner

To better understand the relationship between Ca\textsuperscript{2+} surges and Cl\textsuperscript{-} flux across the membrane, MQAE intensity was used to monitor intracellular Cl\textsuperscript{-} concentrations ([Cl\textsuperscript{-}]\textsubscript{i}) following stimulus of a Ca\textsuperscript{2+} surge with ATP. HEK293 cells stimulated with 50 μM ATP exhibited an increase in [Cl\textsuperscript{-}]\textsubscript{i} (2.76±0.16) that is diminished following Ano1 blockade (1.97±0.23, Figure 4.4). These data indicate that upon [Ca\textsuperscript{2+}]\textsubscript{i} increase Ano1 channels are activated and facilitate a net influx of Cl\textsuperscript{-} ions across the cell membrane.

4.4.3 ATP stimulation of Ca\textsuperscript{2+} surges in HEK293 cells is sensitive to ANO1 expression

In order to examine this phenomenon more clearly, two transgenic cell lines were generated with altered expression of the ANO1 gene. HEK293\textsuperscript{mANO1} transiently express mouse anoctamin-1 and exhibit a 4-fold increase of total ANO1 transcript (RQ = 5.75±1.20) compared to untransfected HEK293 cells (RQ = 1.34±0.39). ATP stimulated Ca\textsuperscript{2+} surges in these cells are intensified both in terms of peak amplitude (4.88±0.43) and velocity of Ca\textsuperscript{2+} entry (0.29±0.09 A.U./sec) compared to control (4.02±0.67 & 0.10±0.03 A.U./sec; Figure 4.3 A, A', E & F). HEK293\textsuperscript{ANO1-KO} exhibit a 3-fold reduction in ANO1 expression (RQ = 0.41±0.22) compared to untransfected HEK293 cells. ATP stimulated Ca\textsuperscript{2+} surges in these cells are diminished in terms of peak amplitude (2.52±0.62 A.U.)
but there was no change in the velocity of Ca$^{2+}$ entry (0.16±0.09 A.U./sec) compared to control (Figure 4.3 C, C’, E & F).

4.4.4 Ca$^{2+}$ surges stimulate cellular proliferation in HEK293 cells in an Ano1 dependent manner

Cellular proliferation was assessed by measurement of proliferating cell nuclear antigen (PCNA) protein expression by ELISA in serum starved cells. ATP stimulation increased PCNA expression in HEK293 cells (57.31±34.66% of control) compared to serum starved controls. This effect was not observed in HEK293$^{\text{ANO1-KO}}$ (-1.37±5.38% of control) or HEK293 cells treated with the MEK inhibitor U0126 (4.60±4.43% of control, Figure 4.5). These data indicate that Ca$^{2+}$ surges are sufficient to promote proliferation in serum-starved, non-proliferative cells and that this response is dependent on Ano1 and mediated via Erk1/2 signaling.

4.4.5 Anoctamin-1 is required for Ca$^{2+}$ mediated PKC activation and Erk1/2 phosphorylation

Western blot analysis was used to semi-quantitatively assess phosphorylation of PKC substrates as a measure of PKC activity. It was found that serum starvation reduced PKC activity and this could be rescued by ATP stimulated Ca$^{2+}$ surges. The Ca$^{2+}$ mediated rescue of PKC activity requires functional Ano1 channels.
indicated by an abolished response following either Ano1 blockade or knockout (Figure 4.6A, B, B’). A similar profile was observed when assessing Erk1/2 phosphorylation. Serum starvation reduced levels of phosphorylated Erk1/2 and this response was rescued by stimulation of intracellular Ca\textsuperscript{2+} surges. Again, this rescue was dependent on functional Ano1 channels, as either Ano1 blockade or knockout eliminated the ATP mediated rescue of phosphorylated Erk1/2 (Figure 4.6A, C, C’). Msk1 phosphorylation was unaffected by any treatments (Figure 4.6A, D, D’). Thus, Ano1 is a required intermediate regulator between intracellular Ca\textsuperscript{2+} surges and PKC mediated activation of Erk1/2 signaling.
Figure 4.1: HEK293 cells were loaded with Fluo-4 AM (5μM) when they reached ~80% confluence and imaged in a physiological PBS. Cells were allowed to equilibrate for 5 min in PBS and then imaged under fluorescence microscopy every 5 seconds for 250 seconds. All values were normalized to the initial fluo-4 fluorescence (F₀) to adjust for well to well variation using the following formula F_t/F₀. At 12.5 seconds cells were pulsed with 50μM ATP to stimulate Ca²⁺ entry via P2X receptors. (A) [Ca²⁺]ᵢ peaked at 42.27±10.09 seconds post-ATP stimulation (PAS) exhibiting a 4-fold increase in fluo-4 intensity. Fluo-4 intensity stabilized at ~2.5 minutes PAS but maintained almost a 2-fold increase of baseline [Ca²⁺]ᵢ. Panel B depicts a representative micrograph of HEK293 cells at peak fluorescence following ATP stimulation.
Figure 4.2: Quantitative PCR was used to measure relative abundance of ANO1 transcript in a transient overexpression model and a CRISPR/Cas9 mediated knock out model in HEK293 cells. HEK293 cells exhibit a ~3-fold increase in total ANO1 transcript cells 24-hours following mANO1 transfection with Lipofectamine® 3000 (HEK293\textsuperscript{mANO1}) relative to untransfected HEK293 cells. HEK293 cells exhibited a reduction in ANO1 transcript 2 passages following transfection of CRISPR/Cas9 plasmid cocktail targeting ANO1 with Lipofectamine® 3000 (HEK293\textsuperscript{ANO1-KO}) relative to untransfected HEK293 cells. (* indicates p<0.05 compared to HEK293; n=6 in each group; error bars are standard deviations)
Figure 4.3: HEK293 cells were loaded with Fluo-4 AM and imaged as previously described. Transient overexpression of mANO1 (HEK293mANO1) increases the peak of the intracellular Ca^{2+} surge stimulated by ATP (50µM) and also increases the initial velocity of Ca^{2+} entry (A, E & F). Panel A' depicts a representative micrograph of HEK293mANO1 cells at peak fluorescence following ATP stimulation. Treatment of HEK293 cells with 20µM of the specific anoctamin-1 inhibitor T16a(inh)-A01 prior to stimulation (A01) decreases the peak of the intracellular Ca^{2+} surge stimulated by ATP (50µM) but has no effect on the initial velocity of Ca^{2+} entry (B, E & F). Panel B' depicts a representative micrograph of HEK293 cells treating with the anoctamin-1 inhibitor at peak fluorescence following ATP stimulation. CRISPR/Cas9 mediated knockout of ANO1 in HEK293 cells (HEK293ANO1-KO) decreases the peak of the intracellular Ca^{2+} surge stimulated by ATP (50µM) but has no effect on the initial velocity of Ca^{2+} entry (C, E & F). Panel C' depicts a representative micrograph of HEK293ANO1-KO cells at peak fluorescence following ATP stimulation. When HEK293 cells are incubated and imaged in Cl⁻ Free PBS prior to stimulation (Cl⁻ Free) the peak of the intracellular Ca^{2+} surge stimulated by ATP (50µM) is decreased and there is a trending decrease in the initial velocity of Ca^{2+} entry, although this is not statistically significant (B, E & F). Panel B' depicts a representative micrograph of HEK293 cells treating with the anoctamin-1 inhibitor at peak fluorescence following ATP stimulation. (* indicates p<0.05 compared to control; error bars are standard deviations)
Figure 4.4: Fluctuations in intracellular Cl\textsuperscript{-} concentrations ([Cl\textsuperscript{-}]\textsubscript{i}) were monitored using the Cl\textsuperscript{-} sensitive fluorescent indicator MQAE. All values were normalized to the initial fluo-4 fluorescence (F\textsubscript{0}) to adjust for well to well variation using the following formula F\textsubscript{0}/F\textsubscript{t}. ATP stimulation (50µ) resulted in an increase in [Cl\textsuperscript{-}]\textsubscript{i} and this response was attenuated by T16a(inh)-A01 (20µM) treatment (A). Peak [Cl\textsuperscript{-}]\textsubscript{i} amplitude was decreased in T16a(inh)-A01 treated cells (1.97±0.23, n=7) compared to control cells (2.76±0.16, n=8, B). (* indicates p<0.05 compared to control; error bars are standard deviations)
Figure 4.5: Cellular proliferation was assessed by expression of PCNA by ELISA. HEK293 and HEK293^{ANO1-KO} cells were subjected to serum starvation for 24-hours to sync cell cycles and cells were then pulsed with ATP (50µM) to stimulate intracellular Ca^{2+} surges. PCNA expression was increased in HEK293 cells (57.31±34.66%) compared to control but not in HEK293^{ANO1-KO} cells (-1.37±5.32%) or HEK293 cells pretreated with the MEK inhibitor, u0126 (4.60±4.43%). (* indicates p<0.05 compared to HEK293+50µM ATP; n=6 in each group; error bars are standard deviations)
Figure 4.6: Western blot analysis reveals that PKC substrate phosphorylation can be stimulated in serum starved HEK293 cells via ATP pulse and that this effect is abolished with either pharmacological inhibition of Ano1 or knockout of ANO1 (A, B, B’). ERK1/2 phosphorylation is also sensitive to ATP stimulation in serum starved HEK293 cells in an Ano1 dependant manner (A, C, C’). Phosphorylation of the Erk1/2 sensitive transcription factor Msk1 is not affected by serum starvation or ATP stimulation (A, D, D’). (For B, C & D: * indicates p<0.05 compared No Treatment Control; For B’, C’ & D’: * indicates p<0.05 compared Serum Starved and ^ indicated p<0.05 compared to SS+ATP; n=6 in each group; error bars are standard deviations)
4.5 DISCUSSION

The intermediary mechanisms that translate Ano1 activity into a discernable signal stimulating Erk1/2 phosphorylation and subsequent cellular proliferation have remained elusive until now. We have shown that intracellular Ca$^{2+}$ surges are amplified by Ano1 (Figure 2) and that this Ca$^{2+}$ activation is a function of Ano1’s Cl$^{-}$ channel activity (Figure 3). Rapid increases in [Ca$^{2+}$]$_{i}$ were sufficient to stimulate activity of PKC and phosphorylation of Erk1/2 in an Ano1 dependent manner (Figure 6). Lastly, [Ca$^{2+}$]$_{i}$ surges are adequate to stimulate cellular proliferation and this response depends upon intact Ano1 activity coupled with Erk1/2 signaling (Figure 5). Thus, we propose the following working model: Ano1 is activated following an initial surge of [Ca$^{2+}$]$_{i}$ acting as a counter-current ion channel to offset local fluctuations in membrane potential and consequently amplifying the original Ca$^{2+}$ signal. Subsequent and enhanced activation of the Erk1/2 signaling cascade via PKC then stimulates cellular proliferation (Figure 4.7).

Our understanding of chloride channel signaling in the process of cellular proliferation has grown significantly although most of this work has focused on the cell volume regulatory function of these channels (Wondergem, Gong et al. 2001, Wang, Wang et al. 2002, Tao, Lau et al. 2008, Habela, Ernest et al. 2009). Ano1 was first identified as an overexpression marker for several cancer types (Katoh and Katoh 2004, West, Corless et al. 2004, Huang, Godfrey et al. 2006).
Since its discovery as the source of the Ca\textsuperscript{2+} activated Cl\textsuperscript{-} current in 2008, Ano1 has been presented as a possible regulator of cellular proliferation in multiple biological contexts (Stanich, Gibbons et al. 2011, Wang, Yang et al. 2012, Qu, Yao et al. 2014, Deng, Yang et al. 2016). Although the precise mechanisms involved in manifesting Ano1’s influence on proliferation may differ between biological systems, it is likely that Ca\textsuperscript{2+} signaling is necessary in this process. For example, Britschgi, et al. found that Ano1 promotes proliferation in breast cancer cell lines via activation of the Ca\textsuperscript{2+} sensitive kinase CAMKII and Akt signaling (Britschgi, Bill et al. 2013). Similar to the model presented in this paper, several biological systems suggest that Ano1’s influence on cellular proliferation is manifested through modulation of the Erk1/2 signaling cascade (Duvvuri, Shiwarshi et al. 2012, Britschgi, Bill et al. 2013, Deng, Yang et al. 2016).

Ca\textsuperscript{2+} is a potent second messenger molecule that is responsible for the regulation of a multitude of critical cellular functions (Clapham 2007). Therefore, tight regulation of intracellular concentrations of intracellular free Ca\textsuperscript{2+} is paramount in regard to both amplitude and duration of [Ca\textsuperscript{2+}]\textsubscript{i} surges. In this study Ca\textsuperscript{2+} entry was stimulated in HEK293 cells via extracellular ATP at an extracellular concentration of 50 μM. Extracellular ATP activates plasma membrane P\textsubscript{2}-type proprioceptors. P\textsubscript{2}-type receptors can be divided into 2 classes, P\textsubscript{2}X receptors are ATP sensitive ligand gated cation channels that stimulate Ca\textsuperscript{2+} entry form the extracellular space and P\textsubscript{2}Y receptors are G-protein coupled ATP receptors that stimulate IP\textsubscript{3} production via PLC which initiates release of Ca\textsuperscript{2+} from intracellular
stores. Subunits of each class are endogenously expressed in HEK293 cells (Schachter, Sromek et al. 1997, Uhlen, Fagerberg et al. 2015). The data presented in this study suggest that the anoctamin 1 Ca$^{2+}$ activated chloride channel amplifies the initial Ca$^{2+}$ entry phase of ATP induced [Ca$^{2+}$]$_i$ surges but had no effect on the rate of [Ca$^{2+}$]$_i$ depletion. This data supports the hypothesis that Ano1’s influence on [Ca$^{2+}$]$_i$ surges is due to activity as a counter current ion channel since the entry phase consist of passive flow of Ca$^{2+}$ into the cytosol that depends on electrochemical gradients. On the other hand, the depletion phase is driven by plasma membrane Ca$^{2+}$ ATPase (PMCA) and sacro/endoplasmic reticulum Ca$^{2+}$ ATPase (SERCA) which pump Ca$^{2+}$ out of the cell or sequester it into intracellular stores in a manner that does not depend on electrochemical gradients. Thus, we have presented a novel physiological mechanism responsible for regulating intracellular Ca$^{2+}$ concentrations which based on the diverse array of intracellular signaling influenced by Ca$^{2+}$ could have far reaching implications.

We also show that stimulation of Ca$^{2+}$ entry by ATP results in an increase in PKC activity and that this increase in dependent on functional Ano1 channels. PKC activation in response to increases in intracellular free Ca$^{2+}$ is well documented and sustained activation of PKC in response to Ca$^{2+}$ has been proposed as essential for mediating long term cellular responses to transient surges in [Ca$^{2+}$]$_i$. Data presented in this study support this theory since serum starved cells exhibit increased PKC activation 24 hours following induction of [Ca$^{2+}$]$_i$ surges in a
manner that is dependent on the function of Ano1 channels. Sustained PKC activation is associated with activation of the Erk1/2 signaling pathway. PKC can activate Erk1/2 signaling through direct phosphorylation of Raf-1, phosphorylation of Raf Kinase Inhibitory Protein (RKIP) that results in its inactivation, or by indirectly activating Ras via Pyk2 phosphorylation (Lev, Moreno et al. 1995, Rikitake, Kawashima et al. 2001, Corbit, Trakul et al. 2003, McCubrey, Steelman et al. 2007). Here we have described one of many possible intracellular signaling mechanisms that may be sensitive to Ano1 and its influence on intracellular Ca\(^{2+}\) fluctuations.

Cells with more negative resting membrane potentials (muscle cells and neurons) initiate Cl\(^{-}\) efflux upon activation of Ano1. Spontaneously Hypertensive Rats (SHR) overexpress Ano1 in vascular smooth muscle cells (VSMC) compared to the non-hypertensive Wistar-Kyoto Rat (WKY). Overexpression of Ano1 elevates vascular tone and increases blood pressure due to elevated total peripheral resistance. The hypertension in SHR is subsequently dependent upon Ano1 mediated increases in [Ca\(^{2+}\)]. An alternative mechanism has been described in VSMCs of SHR that allows for amplified Ca\(^{2+}\) signaling despite the resultant Cl\(^{-}\) efflux of Ano1 activation. It is proposed that upon activation of voltage dependent Ca\(^{2+}\) channels Ano1 is activated and the resultant Cl\(^{-}\) efflux further depolarizes the membrane leading to further activation of voltage dependent Ca\(^{2+}\) and amplified [Ca\(^{2+}\)]. (Wang, Li et al. 2015). This speaks to the versatility of Ano1’s influence on [Ca\(^{2+}\)] and begins to elucidate its involvement in
multiple pathological and physiological processes across multiple diverse cell types.

In conclusion, we identified that Ano1 acts as a positive feedback mechanism by amplifying \([\text{Ca}^{2+}]\) in non-excitable cells when activated by initial increases in \([\text{Ca}^{2+}]\). This is accomplished through its activity as a counter current ion channel where local disturbances in \(V_{\text{mem}}\) caused by initial \(\text{Ca}^{2+}\) entry are offset by \(\text{Cl}^{-}\) entry. Furthermore, we identified that this \([\text{Ca}^{2+}]\) amplification was sufficient to stimulate Erk1/2 signaling and subsequent cellular proliferation. These findings have far reaching implications considering the ubiquitous nature of Ano1 expression and the diversity of intracellular \(\text{Ca}^{2+}\) signaling. This is especially true considering the multiple physiological and pathophysiological functions already discovered in brief history following Ano1’s discovery as a CaCC.
Figure 4.7: This represents the proposed model explaining the mechanisms mediating Ano1 influence on intracellular Ca$^{2+}$ surges and how this results in activation of p44/42 MAPK signaling and proliferation.
4.6 Acknowledgements

This work was funded by the Biological Sciences Research Group, LLC.
CHAPTER 5
GENERAL CONCLUSIONS AND DISCUSSION
Brandon Michael Franklin

**Abbreviations used:** Kv (voltage-gated potassium channels), Ca$^{2+}$ (calcium cation), TNF-α (tumor necrosis factor alpha), IL-8 (interleukin 8), 4-AP (4-aminopyridine), $V_{\text{mem}}$ (plasma membrane potential), MAPK (mitogen activated protein kinase), Ano1 (anoctamin 1), Erk1/2 (extracellular signal regulated kinase 1/2), Cl$^{-}$ (chloride anion), CAMKII (calmodulin-dependent protein kinase II), Akt (protein kinase B), [Ca$^{2+}$] (intracellular Ca$^{2+}$ concentrations), ICC (interstitial cells of Cajal), Ano2 (anoctamin 2), Ano6 (anoctamin 6), Kv (voltage-gated potassium channels), Cav (voltage-gated calcium channels), GlyR (glycine receptor), GABA$\alpha$R (GABA receptor type A), SERCA (sarcoplasmic/endoplasmic reticulum calcium ATPase)

**Keywords:** Ions, Ion channels, Electric fields, Wound-healing, Regeneration
5.1 Influence of Kv Channels on Phagocyte Activity during Larval Tail Regeneration and in Response to Exogenous Electrical Stimulation

The data presented in this dissertation regarding Kv channel activation of immune cells represent two models from vastly different biological context. Amphibian tail regeneration in the vertebrate, *Ambystoma mexicanum* and the soft tissue response of exogenous electrical stimulation in the invertebrate *Procambarus clarkii*. These two systems have vastly divergent immune systems with the former made up of the lymphocytes and macrophages characteristic of higher vertebrates and the latter consisting of the more primitive hemocyte lineages. However, hemocytes do share some homology with macrophages both in terms of phagocytic function and morphology (Hose, Martin et al. 1990, Söderhäll, Bangyeekhun et al. 2003). Here I have shown that the phagocytic cell dependence on functional Kv channels is conserved between these two evolutionarily diverged species in the context of their response to either exogenously applied electrical fields or in the physiological response to amputation. This is not surprising considering the conservation of structure and function observed in Kv channels from prokaryotic and eukaryotic systems (Doyle, Cabral et al. 1998).

The fact that Kv channels play a physiological role during the activation and function of immune cells has been known for some time with the earliest studies
focused on T lymphocytes and their role in regulating membrane potential and Ca^{2+} flux (Lewis and Cahalan 1995, Cahalan and Chandy 2009). More recently a similar role has emerged in macrophages with KV channels influencing a multitude of macrophage functions. KV channel function has been demonstrated in regulating both the proliferation of macrophages and their ability to recruit the macrophage precursor Ly6C monocytes (Zhang, Wang et al. 2015). KV channels are also important regulators of cytokine production in macrophages indicated by a reduction of both TNF-α and IL-8 production in human macrophages in response to blockade of KV channels with 4-AP (Qiu, Campbell et al. 2002). It is known that macrophages alter the electrophysiological properties of their membranes dependent on their activation state and the resultant modulation of ion movement across the membrane is important for regulating their function (Eder 1998, Vicente, Escalada et al. 2003). At the molecular level, modulated expression patterns of KV channel subunits have been demonstrated in response to experimentally induced activation (Villalonga, David et al. 2010). Taken together these data suggest that KV channels are important and dynamic regulators of $V_{\text{mem}}$ and Ca^{2+} handling in macrophages and that these properties are important physiological regulators of macrophage activation and function.

Macrophages are important components in the tissue response to injury. An early and important role of macrophages in the wound healing process is to clean up the wounded area by phagocytosis of cellular debris and apoptotic cells to
create a permissive environment for wound healing (Peiser, Mukhopadhyay et al. 2002). They also act as the cellular initiators of the initial response to injury providing a source for important inflammatory signals that initiate the wound response in fibrotic wound healing (Duffield, Forbes et al. 2005, Wynn and Barron 2010). Following the initial inflammatory response to wounding, macrophages exhibit a phenotypic switch and begin to produce angiogenic and proliferation inducing growth factors (Willenborg, Lucas et al. 2012, Wynn and Vannella 2016). Interestingly, macrophages are also critical to the non-fibrotic regenerative response to wounding in axolotls indicated by failure to regenerate amputated limbs following depletion of endogenous macrophage populations (Godwin, Pinto et al. 2013). This suggests that there is differential regulation of macrophage function in response to wounding in regenerating versus non-regenerating systems where the fibrotic response is lost but the tissue repair signals are conserved. Perhaps a better understanding of how to control macrophage function through control of $V_{\text{mem}}$ and $K_v$ channel function could lead to novel therapeutic techniques that produce a more efficient and reparative wound healing response in humans.
5.2 Anoctamins and their Role in Regulating the p44/42 MAPK Pathway via Control of Ca\(^{2+}\) Signaling and Homeostasis

The data presented in this dissertation outline an important role for Ano1 in regulating the critical proliferation response during the onset of amphibian tail regeneration. I have also shown that this regulatory control of proliferation by Ano1 is conserved in mammalian cells and outlined the specific signaling components sensitive to Ano1 function in this system. In that regard, I have identified a novel physiological mechanism important for the regulation of Ca\(^{2+}\) signaling in mammalian cells that is dependent on Ano1’s function as a Cl\(^{-}\) channel. These data suggest that Ano1 is involved in amplifying Ca\(^{2+}\) transients by a positive feedback mechanism following an initial surge of Ca\(^{2+}\) that activates Ano1’s Cl\(^{-}\) channel function. Subsequently this amplified Ca\(^{2+}\) signal stimulates the versatile Erk1/2 signaling pathway and cellular proliferation. Thus, Ano1 is a critical regulator of proliferation in a model of amphibian regeneration and the relevant physiological function and signaling mechanisms appear to be conserved in mammalian systems. This demonstrates that Ano1 may be a novel therapeutic target to enhance human wound healing and tissue repair.

Anoctamin 1 has been implicated as an important regulator of cellular proliferation in the physiological life and death cycle of cells in the gastrointestinal system, during tissue repair following injury and in the pathogenesis of several
types of cancer (Stanich, Gibbons et al. 2011, Qu, Yao et al. 2014). In these particular tissues, the influence of Ano1 on cellular proliferation is manifested through modulation of the Erk1/2 signaling cascade (Duvvuri, Shiwarski et al. 2012, Britschgi, Bill et al. 2013, Deng, Yang et al. 2016).

Our understanding of chloride channel signaling in the process of cellular proliferation has grown significantly although most of this work has focused on the cell volume regulatory function of these channels (Wondergem, Gong et al. 2001, Wang, Wang et al. 2002, Tao, Lau et al. 2008, Habela, Ernest et al. 2009). Ano1 channels were first identified as an overexpression marker for several cancer types (Katoh and Katoh 2004, West, Corless et al. 2004, Huang, Godfrey et al. 2006). Since its discovery as the source of the Ca^{2+} activated Cl^{-} current in 2008, Ano1 channels have been presented as a possible regulator of cellular proliferation in multiple biological contexts (Stanich, Gibbons et al. 2011, Wang, Yang et al. 2012, Qu, Yao et al. 2014, Deng, Yang et al. 2016). Although the precise mechanisms involved in manifesting Ano1’s influence on proliferation may differ between biological systems it is likely that Ca^{2+} signaling is necessary in this process. For example, Britschgi, et al. found that Ano1 channels promote proliferation in breast cancer cell lines via activation of CAMKII and Akt signaling (Britschgi, Bill et al. 2013). While the terminal signaling cascades may differ between this model and ours, the Ano1 mediated amplification of [Ca^{2+}]_{i} is likely responsible for the activation of CAMKII.
While much of the interest in Ano1 has focused on its roles in normal cellular proliferation and the abnormal development of cell division in cancer, it is an ion channel that is expressed in a ubiquitous manner with documented expression in a variety of epithelial, smooth muscle, neuronal, renal, endocrine and reproductive tissues (Duran and Hartzell 2011). In these diverse tissue types, Ano1 function has been equated to multiple pathological and physiological functions. In renal tissues, cellular proton secretion and protein reabsorption in proximal tubular cells is stimulated by Ano1 function. Ano1 overexpression is associated with the formation of cysts in polycystic kidney disease (Buchholz, Faria et al. 2014, Faria, Rock et al. 2014). Ano1 expression activates the slow wave electrical potential of gastrointestinal smooth muscle interstitial cells of Cajal (ICC) (Hwang, Blair et al. 2009). Ano1 has also been implicated in nociception and heat sensing in sensory neurons (Cho, Yang et al. 2012). While these examples of Ano1 function and dysfunction are not all encompassing, they indicate the diversity of its function and the scientific importance of developing an intimate understanding of the molecular mechanisms mediating the influence of Ano1 channel function in regulating these diverse processes.

The anoctamin family of proteins have garnered much interest in recent years. Following the identification of Ano1 and Ano2 as the molecular determinant of the calcium activated chloride current they have been vigorously investigated
(Kotecha and Schlichter 1999, Caputo, Caci et al. 2008, Schroeder, Cheng et al. 2008, Yang, Cho et al. 2008). There are 10 members of the anoctamin family (Ano1-10) and all of them have eight transmembrane domains and all have been proposed to have ion channel functionality there are differing reports on this (Milenkovic, Brockmann et al. 2010, Tian, Schreiber et al. 2012). Cl⁻ channel activity is stimulated by Ca²⁺ in both Ano1 and Ano2 although Ano2 is less sensitive to Ca²⁺ and Ano2 activation and deactivation kinetics are much faster compared to Ano1 (Caputo, Caci et al. 2008, Schroeder, Cheng et al. 2008, Yang, Cho et al. 2008, Pifferi, Dibattista et al. 2009). The Cl⁻ channel activity of Ano3-10 is less certain. Ano6 for example has been proposed to have function as both an ion channel and a phospholipid scramblase that may influence the activity of other channels but whether the scramblase and channel activity of Ano6 are mutually exclusive or codependent on each other are unclear (Scudieri, Caci et al. 2015). Furthermore, the ion selectivity of Ano6 is a debated topic with conflicting reports that it is a Ca²⁺ sensitive Cl⁻ channel and on the other hand is a Ca²⁺ sensitive Ca²⁺ channel (Duran and Hartzell 2011). With multiple implicated functions as plasma membrane ion channels and scramblases and implications of multiple anoctamin family members in human health and disease it will be interesting to further elucidate the function of this family of proteins.
5.3 Summary

In this dissertation I have discussed the importance of ion channels in the context of wound healing and regeneration and specifically their ability to influence critical cellular processes of non-excitabile cells that are required for these events. Ion channels are diverse and versatile proteins that are important for the physiological function of cells dating back to the earliest forms of life. I have presented data in this dissertation that implicates a role for multiple ion channels in regulating functions such as immune cell activation and progenitor cell proliferation. These are important aspects of tissue repair and the data I have presented in this dissertation suggest that ion channel regulation of these cellular events evolved early in the tree of life and have been conserved from invertebrate to vertebrate species. Although higher vertebrates have a reduced capacity for tissue regeneration, the ability of ion channels (specifically Ano1) to influence critical cellular processes necessary for the more robust regenerative processes observed in axolotls appear to be intact. This suggests that ion channels represent potential therapeutic targets for novel clinical therapies aimed at enhancing human wound healing and tissue regeneration.

Chapter 3 of this dissertation outlined a chemical genetic screen that identified the following ion channels as necessary for axolotl regeneration: anoctamin1/Tmem16a, anoctamin2/Tmem16b, Kv2.1, Kv2.2, L-type Cav channels, H/K ATPases, GlyR, GABA\_\alpha R, Kv1.5 and SERCA pumps.
Furthermore, I have indicated a role for Ano1 in regulating proliferation via Ca\textsuperscript{2+} and Erk1/2 signaling, a function that appears to be conserved in human cells. I presented additional data that indicated Kv channels as important to the necessary function of immune cells required during regeneration in axolotl. Future studies should focus on further elucidating the role of Kv channels in regulating macrophage activities during axolotl regeneration and how/if Kv channel function may be modulated in higher vertebrates to influence macrophage activity and enhance tissue repair. Additionally, it will be interesting to increase our understanding of the other channels identified in this screen in regards to their involvement in axolotl regeneration and whether or not these channel functions are conserved in humans and thus represent novel therapeutic targets to enhance human tissue repair.

Chapter 4 was aimed at deeper investigation of the role of Ano1 in regulation of cellular proliferation and whether or not this function was conserved in human cells. In this work, I have described a novel physiological regulator of intracellular calcium signaling in non-excitable cells. The implications of this discovery could be far reaching. As discussed earlier, Ca\textsuperscript{2+} signaling is a versatile and diverse signaling mechanism that is important to virtually all cell and tissue types. Most relevant to this dissertation, Ano1 represents a key component involved in driving cellular proliferation during tissue regeneration. Taken together with the fact that the relationship between Ano1 and intercellular Ca\textsuperscript{2+} signaling identified in the amphibian model of regeneration in chapter 3 are
conserved in human cells, modulation of Ano1 expression and function may have potential to enhance human wound healing and regeneration. This new understanding of the function of Ano1 and its role as a counter current chloride channel that acts to amplify calcium signaling stretch beyond the realms of wound healing and regeneration. We know that the pathophysiological generation of several diseases are associated with overexpression or over activity of Ano1 channels. Future studies should investigate the role of Ano1 mediated control of intracellular Ca\textsuperscript{2+} signaling in these models and assess the viability of Ano1 as a therapeutic target for human health and disease.
APPENDIX 1

OPEN PROBLEM-BASED INSTRUCTION IMPACTS UNDERSTANDING OF PHYSIOLOGICAL CONCEPTS DIFFERENTLY IN UNDERGRADUATE STUDENTS

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¹Department of Biology, University of Kentucky, Lexington KY

Abbreviations used: LI (traditional lecture-style instruction), GPBI (guided problem-based instruction), OPBI (open problem-based instruction), MC (multiple choice), SA (short answer), PBL (problem-based learning), GIL (guided inquiry-based learning), GPA (grade point average), BIO (biology), ANOVA (analysis of variance), SNK (Student-Newman-Keuls), STEM (science, technology, engineering and math), NSF (national science foundation)

Keywords: Active learning, problem based learning, scaffolding, performance gaps

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A1.1 Abstract

Student populations are diverse such that different types of learners struggle with traditional didactic instruction. Problem-based learning has existed for several decades but there is still controversy regarding the optimal mode of instruction to ensure success at all levels of students’ past achievement. This study addressed this problem by dividing students into three instructional groups for an upper-level course in animal physiology: traditional lecture-style instruction (LI), guided problem-based instruction (GPBI) and open problem-based instruction (OPBI). Student performance was measured by three summative assessments consisting of 50% multiple choice (MC) questions and 50% short-answer questions (SA) as well as a final overall course assessment. This study also examined how students of different academic achievement histories performed under each instructional method. When not considering student achievement levels, the effects of instructional methods on student outcomes were modest; OPBI students performed moderately better on SA exam questions than both LI and GPBI groups. High-achieving students showed no difference in performance for any of the instructional methods on any metric examined. In students with low-achieving academic histories, OPBI students largely out-performed LI students on all metrics (SA-exam: $p<0.05, d=1.865$; MC-exam: $p<0.05, d=1.166$ and Final score: $p<0.05, d=1.265$). They also out-performed GPBI students on short-answer exam questions ($p<0.05, d=1.109$) but not MC exam questions ($p=0.071, d=0.716$) or final course outcome ($p=0.328, d=0.513$). These findings strongly suggest that typically low-achieving students perform at a higher level
under OPBI instruction as long as the proper support systems (formative assessment and scaffolding) are provided to encourage student success.
A1.2 Introduction

Several decades ago problem-based learning (PBL) was introduced at McMaster University Medical School, and it challenged the fundamental pedagogical principles of science education and the teaching of physiology (Barrows 1980). The success and popularity of PBL in medical education was noticed in the community of undergraduate education where its methods were adapted to this student population (Woods 1994, Wilkerson and Gijselaers 1996). This movement of teaching through problem solving resurrected the closely related method of guided inquiry-based learning (GIL), which conveys information to students through the process of scientific inquiry under more direct instructor supervision and guidance (Farrell, Moog et al. 1999, Kuhn, Black et al. 2000). Both PBL and GIL are rooted in the social constructivist theories of Dewey (Dewey 2007) and both follow a cyclical process that reinforces and builds upon information throughout the semester. Students are presented with a problem or question and a set of “what is known” data from which they will generate a hypothesis. At this point the students are given new data and asked to use critical reasoning skills to rethink their original hypothesis and discuss it with the class. This process is dually beneficial for the students, they gain knowledge of the concepts discussed and develop skills in hypothesis generation and critical reasoning that develop throughout the semester and can be used throughout their career (Figure A1.1). These methods are successful because many
learners don’t maximally benefit from traditional didactic instructional methods and instead prefer a more active learning style (Spencer 1999).

Recently, controversy has arisen regarding the effectiveness of PBL and GIL, labeling them as minimal guidance instructional methods (Kirschner, Sweller et al. 2006). These arguments possess two potentially fatal flaws. First, while these methods may vary in their level of instruction, there is always some degree of direct instruction coupled with significant scaffolding for student instructional support (Schmidt 1983, Barrows 1986, Hmelo-Silver, Duncan et al. 2007). Secondly, the study in question selectively identified literature that supports his claims while ignoring the wealth of evidence to the contrary (Kirschner, Sweller et al. 2006, Hmelo-Silver, Duncan et al. 2007). There exists a significant archive of literature that strongly supports and advocates for the efficacy of PBL and GIL in medical school, university and secondary-school student populations (Hmelo 1998, Dochy, Segers et al. 2003, Mergendoller, Maxwell et al. 2006, Koh, Khoo et al. 2008, Schmidt, Van der Molen et al. 2009, Strobel and van Barneveld 2009, Minner, Levy et al. 2010). There is a paucity of data however, that addresses the specific level or degree of guidance best suited for these types of constructivist, discovery-based learning environments.

To address this, a required undergraduate core physiology course was designed with three different levels of instruction and predictions were made about potential student outcomes for each mode of instruction (Table A1.1). Traditional lecture-
style instruction (LI) has been proven to effectively transmit both knowledge and understanding to students but falls short in successfully training students with sufficient problem solving skills (Dochy, Segers et al. 2003, Şendağ and Odabaşı 2009). Constructivist learning models have been shown to be equally effective at conveying knowledge and understanding while also improving critical thinking skills and self-directed learning (Hmelo 1998, Hmelo and Lin 2000, Koh, Khoo et al. 2008, Loyens, Magda et al. 2008, Schmidt, Rotgans et al. 2011). These previous studies however, do not address how these methods work on different populations of students (i.e. low and high-achieving students) and whether different levels of guidance, within a constructivist model, will affect these populations differently.

This present study investigated how undergraduate students, with different levels of overall academic history responded to different modes of instruction in a required upper-level core animal physiology course. Both declarative knowledge and critical thinking skills were assessed, by multiple-choice (MC) exam questions and short-answer (SA) exam questions, respectively.
Figure 1: Problem-based learning is a cyclical process where practices and concepts learned in one exercise must be reused and reinforced in subsequent activities.
<table>
<thead>
<tr>
<th>Instruction</th>
<th>Lecture-Style Instruction (LI)</th>
<th>Guided Problem-Based Instruction (GPBI)</th>
<th>Open Problem-Based Instruction (OPBI)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>• Traditional lecture-style delivery of material</td>
<td>• A combination of instructor-directed and student-directed learning</td>
<td>• Student-directed learning</td>
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<td></td>
<td>• Hands-on laboratory to reinforce core concepts</td>
<td>• The instructor is more hands-on, providing content to students and providing unsolicited guidance during their problem solving activities</td>
<td>• The instructor is more hands-on, providing guidance to students only when requested</td>
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<td></td>
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<td>• Use of formative assessment to identify problem areas for improvement</td>
<td>• Use of formative assessment to identify problem areas for improvement</td>
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<td></td>
<td></td>
<td>• Hands-on laboratory to reinforce core concepts</td>
<td>• Hands-on laboratory to reinforce core concepts</td>
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<tr>
<td>Assumptions about Learning</td>
<td>Students benefit most from systematic dissemination of content by an expert.</td>
<td>Students will benefit most from problem solving exercises if the necessary information is given to them by an instructor and they are guided through the problem solving process.</td>
<td>Students will benefit most if challenged to seek out the information needed to solve real-world problems.</td>
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<tr>
<td>Motivations for Learning</td>
<td>Performance on exams</td>
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<td>Overall course score</td>
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<td>Students are more motivated to study before class because of a desire to contribute meaningfully to their peer groups</td>
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<tr>
<td>Assessment</td>
<td>Summative assessment: 3 exams consisting of both multiple choice and short answer questions. 5 writing assignments stemming from the laboratory portion on the course.</td>
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<td></td>
<td>Formative assessment: none</td>
<td>Student progress monitored on a daily basis through low-stakes in-class assignments and mini-presentations</td>
<td>Student progress monitored on a daily basis through low-stakes in-class assignments and mini-presentations</td>
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<tr>
<td>Potential Outcomes</td>
<td>Content acquisition and understanding</td>
<td>Critical thinking and problem-solving skills</td>
<td>Content acquisition and understanding</td>
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<td>Hypothesis generation, data analysis and interpretation</td>
<td>Critical thinking and problem-solving skills</td>
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<td>Hypothesis generation, data analysis and interpretation</td>
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<td>Effective self-directed learning skills</td>
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<td>Ability to structure knowledge to efficiently solve a problem</td>
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Table 1: Characterization and potential outcomes of each teaching method.
A1.3 Methods and Materials

A1.3.1 Instructional Groups

Different instructional groups and instructional methods were developed and labeled as “Lecture Instruction (LI)”, “Guided Problem-Based Instruction (GPBI)” and “Open Problem-Based Instruction (OPBI)”. All students randomly selected the different instructional groups based upon their time and availability of course scheduling and were unaware of the type of instruction offered prior to course enrollment. Wet laboratory instruction was also provided for all students and the laboratory style instruction was not different for all groups. To control for classroom instructional ability, the same professor designed each instructional session and conducted the teaching for all sessions. Student cumulative GPAs were not different between instructional groups at the onset of the course (p>0.05). All course materials such as recommended texts, access to power point materials, supplemental reading, website and laboratory materials were identical for all groups of students in the study.

A1.3.1.1 Lecture-style instruction (LI)

This group consisted of 47 biology majors with a cumulative GPA of 3.29±0.53. These students were presented with information through traditional lecture-style
classroom instruction by a full professor in the University of Kentucky’s department of biology during 75 minute instructional sessions held on Tuesday and Thursday of the 13 week semester.

A1.3.1.2 Guided problem-based instruction (GPBI)

This group consisted of 38 biology majors with a cumulative GPA of 3.25±0.54. Instructional times for these students consisted of 75-minute session that met two times weekly (Tuesday and Thursday) over the 13 week semester. During class periods, students worked in small, randomly formed groups of 3-4 on guided activities and short time frame tutorials (8-10 minutes) designed to encompass the same concepts covered in the LI group. The instructor of these sessions had a significant role as a facilitator, providing selected content to students at the onset of the lesson and providing unsolicited guidance during their problem solving activities.

A1.3.1.3 Open problem-based instruction (OPBI)

This group contained 35 biology majors with a cumulative GPA of 3.40±0.53. The instructional design of this group was somewhat similar to the GPBI group but with
marked and significant differences. Students randomly chose to work jointly in
small groups of 3-4 on similar and sometimes the same activities provided to the
GPBI instructional group. The major instructional difference of the OPBI group
from the GPBI group is that no formal dissemination of information and guidance
was provided during the initiation of OPBI problem solving activities. Students
were provided with a core question or series of core questions to begin the
instruction. Information then was provided when solicited by the students in
working groups and was provided in a manner that directed students to explore
and seek the type of information required for problem solving activities and core
questions.

A1.3.2 Randomization of Subjects and Grading

All students enrolling in BIO 350: Animal Physiology at the University of
Kentucky chose one of seven sections. Each section had been assigned one of
three instructional treatment groups (LI – 3 sections; GPBI and OPBI – 2 sections
each). During the enrollment process students were unaware that different
teaching methods would be used in different sections. At the beginning of the
course, all of the students were informed of the study and completed an informed
consent. Once informed, students were given the opportunity to switch sections if
they preferred an alternate teaching methodology, although no students exercised
this option.
All exams and assignments were graded blindly. Multiple choice (MC) questions were graded by Scantron® scanners and entered into a database by a graduate teaching assistant. Short answer (SA) questions were all graded by the professor in charge of the course. Student names and section numbers were replaced with an unidentifiable 8-digit code on all exam forms and exams were shuffled so that they were in a random order and unidentifiable by instructional group. Answer rubrics were generated for each SA question and questions were graded in numerical order with each question for all exams completely graded before advancing to the next question to be graded. Upon completion of grading, a graduate teaching assistant re-sorted the exams by section number, decoded student names and entered scores into a database.

A1.3.3 Assessment

A1.3.3.1 Summative assessment

Student performance was summatively assessed by three exams that were 50% MC and 50% SA questions. Students from all three instructional groups (LI, GPBI & OPBI) took the same exams on the same days. On exams 1 and 2 there were 60 possible points for MC and 65 possible points for SA. Exam 3 had 80 possible points for both SA and MC. The concepts covered on each exam are depicted and
described in Table A1.2. Exam 1 focused primarily on cell physiology, membranes and membrane potentials and neuronal function. Exam 2 was the largest unit in terms of overall physiological content and concepts. This unit consisted of skeletal muscle contraction, cardiovascular, renal and blood pressure control. In exam 3, the major focus was on respiration, acid-base balance and endocrinology and reproductive physiology. All exams consisted of 1/3 Bloom’s taxonomy level 1/2 questions, 1/3 Bloom’s level 4/5 questions and 1/3 Bloom’s level 8 or greater questions. The week prior to final exams consisted of a short discussion of digestion and a more complete component of nutrient and energy balance. It is important to note that all exams were cumulative and included conceptual aspects from previous units. This method of assessment was utilized to support the different instructional modalities thereby forcing students to continually incorporate conceptual understandings of physiology in a holistic manner.

A1.1.3.3.2 Formative assessment

The LI group had no ongoing or specific formative assessment conducted between exams. In the GPBI and OPBI groups, students engaged in daily, non-graded activities and class discussions. Activities and class discussions by group did allow the instructor significant indications of conceptual areas requiring reinforcement or in some cases further instruction.
A1.3.4 Identification of Academic Performance Groups

To investigate the influence of these different instructional methods on physiology students with different overall academic proficiencies, students were divided into three subgroups within each instructional group (Table A1.3). High-achieving students were defined as students with a cumulative GPA of 3.6 or higher at the onset of the course, average-level students began the course with a cumulative GPA between 3.0 and 3.6 and low-achieving students were defined as students with a cumulative GPA of 3.0 or less at course onset. Students of all achievement levels were integrated into each instructional group and received the same instruction.

A1.3.5 Statistical Analysis

Exam and final course score are presented as mean ± standard error of total points (pts) earned. When comparing LI, GPBI and OPBI or high, average- and low-achieving students, we used either one-way ANOVA with Student–Newman–Keuls (SNK) post hoc analysis (for normally distributed data) or Kruskal-Wallis ANOVA on ranks with Dunn’s test for post hoc analysis (for data not normally distributed). Effect size between groups was calculated with Cohen’s $d$. All data are presented
as mean ± standard error, unless otherwise noted. The 0.05 level of probability was utilized as the criterion for significance in all data sets.
<table>
<thead>
<tr>
<th>Exam 1</th>
<th>Exam 2</th>
<th>Exam 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Feedback control systems</td>
<td>All previous material</td>
<td>All previous material</td>
</tr>
<tr>
<td>Membranes, channels &amp; transport</td>
<td>Neuromuscular junction/function</td>
<td>Respiration</td>
</tr>
<tr>
<td>Neuronal biophysics</td>
<td>Muscle contraction (skeletal, cardiac &amp; smooth)</td>
<td>Acid-base balance</td>
</tr>
<tr>
<td>Neuronal transmission</td>
<td>Cardiovascular function</td>
<td>Endocrine function</td>
</tr>
<tr>
<td>Neuronal integration</td>
<td>Renal function</td>
<td>Sexual reproduction</td>
</tr>
<tr>
<td>CNS &amp; sensory mechanisms</td>
<td>Blood pressure control</td>
<td>Digestion &amp; nutrient balance</td>
</tr>
<tr>
<td></td>
<td>Ionic &amp; osmotic balance</td>
<td>Energy balance</td>
</tr>
</tbody>
</table>

**Table A1.2:** Physiological concepts covered by each exam.
Table A1.3: Sample sizes for the instructional groups and achievement levels

<table>
<thead>
<tr>
<th></th>
<th>High-Achievers</th>
<th>Average-Achievers</th>
<th>Low-Achievers</th>
</tr>
</thead>
<tbody>
<tr>
<td>LI</td>
<td>14</td>
<td>25</td>
<td>8</td>
</tr>
<tr>
<td>GPBI</td>
<td>10</td>
<td>14</td>
<td>14</td>
</tr>
<tr>
<td>OPBI</td>
<td>18</td>
<td>8</td>
<td>9</td>
</tr>
</tbody>
</table>
A1.4 RESULTS

A1.4.1 General student population exam performance by instructional group

Student performance on MC questions for all three exams and averaged MC scores are summarized in Figure A1.2A. OPBI students scored significantly higher on the MC portion of exam 2 (48.18±1.08 points) compared to GPBI (43.64±1.14 points, p<0.05, d=0.667) or LI (43.75±1.14 points, p<0.05, d=0.617) students. OPBI students tended to perform better on MC portions of other exams but no other statistical significance was found (Figure A1.2A). On the SA portion of exams OPBI students scored better on average (55.50±1.20 points) compared to GPBI (49.79±1.75 points, p<0.05, d=0.619) but not LI (50.46±1.62 points, p=0.070, d=0.540) students (Figure A1.2B). Although there are no statistical differences between LI students and OPBI students on SA exam questions, OPBI students overall do score higher on every exam (Figure A1.2B).

A1.4.2 Effect of PBL on high and low-achieving students’ exam performance

Low-achieving students benefited greatly from the open problem-based style of instruction (Figure A1.3C, A1.3D). They scored better on multiple-choice exam questions when instructed by OPBI (53.33±1.83 points) compared to LI (45.04±2.98 points, p<0.05, d=1.166) but not GPBI (48.19±1.52 points, p=0.071,
\(d=0.716\), although there is a trend for significance. There was an increase in MC score for OPBI (45.56±1.69 points) students compared to both GPBI (38.29±2.08 points, \(p<0.05, d=1.267\)) and LI (38.75±2.00 points, \(p<0.05, d=1.267\)) students on exam 2 (Figure A1.3C). OPBI proved to be of an even greater benefit for low-achieving students on SA exam questions with OPBI students scoring much higher on average (54.63±2.05 points) compared to both LI (38.04±3.88 points, \(p<0.05, d=1.865\)) and GPBI (44.33±3.10 points, \(p<0.05, d=1.109\)) (Figure A1.3D).

When focusing on high-achieving students there were no observable differences on MC exam questions between LI (58.10±1.37 points), GPBI (58.67±0.81 points) or OPBI (60.08±0.95 points) students (Figure A1.3A). These students also exhibited no difference on responses to SA exam questions between LI (55.74±1.60 points), GPBI (57.33±1.32 points) or OPBI (58.83±1.14 points) students (Figure A1.3B).

A1.4.3 Overall course performance

Overall course outcome was improved for low-achieving students instructed by the OPBI method (556.81±20.23 points) compared to LI (450.56±36.08 points, \(p<0.05, d=1.265\)) students (Figure A1.4). In fact, while GPA was a good indicator of overall course performance, with low-achieving students scoring lower (498.38±17.97 points) than both average-level (573.07±9.07 points, \(p<0.05, d=0.979\)) and high achieving (High: 614.30±8.66 points, \(p<0.05, d=0.979\))
$d=0.1547$) students, low-achieving students instructed by the OPBI method (556.81±20.23 points) exhibited an overall course outcome not different from that of average-level ($p=0.315$, $d=0.283$) students (Figure A1.5).
Figure A1.2: Open problem-based instruction (OPBI) had little effect on overall student exam performance compared to either traditional lecture instruction (LI) or guided problem-based instruction (GPBI) groups. SA, short answer. *P < 0.05 compared with the LI group; ^P < 0.05 compared with the GPBI group.
Figure A1.3: High-achieving students performed just as well on both multiple-choice (MC; A) and SA (B) exam questions regardless of instructional group. Low-achieving students in the OPBI group performed better on MC (C) and SA (D) exam questions compared with both LI and GPBI groups. *P < 0.05 compared with the LI group; ^P < 0.05 compared with the GPBI group.
Figure A1.4: Low-achieving students performed better in the course when instructed by either OPBI or GPBI. The maximum possible points are represented by the dashed line. *P < 0.05 compared with the LI group; ^P < 0.05 compared with the GPBI group.
Figure A1.5: Low-achieving students had lower final course scores compared with both high- and average-achieving students. When low-achieving students were instructed by the OPBI method, they performed as well as average-achieving students. The maximum possible points are represented by the dashed line. *P < 0.05 compared with high-achieving students; ^P < 0.05 compared with average-achieving students.
A1.5 Discussion

This study set out to achieve two primary goals. The first was to characterize and describe two distinctive varieties of PBL with differing degrees of direct instruction (Table A1.1). To be clear, neither of these methods, OPBI or GPBI, should be considered minimal guidance methods as there is significant scaffolding in place to ensure student success. The second goal was to evaluate the efficacy of these methods on student populations of diverse academic background, when compared with traditional lecture style instruction. Many studies have found that PBL is a more effective method than LI, particularly when considering critical reasoning skills and long-term knowledge retention (Vernon and Blake 1993, Antepohl and Herzig 1999, McParland, Noble et al. 2004, Strobel and van Barneveld 2009). The results of this study were not different, as students in the PBL groups outperformed LI instructed students on assessment directed at critical reasoning skills (short answer exam questions).

There has been some controversy regarding the effectiveness of constructivist methods and the level of guidance that should accompany them (Kirschner, Sweller et al. 2006, Hmelo-Silver, Duncan et al. 2007). Leppink and colleagues found that the added guidance of a GPBI course was moderately beneficial to novice, entry-level students with little or no prior knowledge of the course content (Leppink, Broers et al. 2014). The present study found the opposite to be true in non-novice students, as the OPBI group performed moderately better than LI.
instructed students on assessments of critical reasoning skills, although GPBI students’ performance was generally not different. That is to say, students who have been exposed to the core concepts in previous introductory courses and have a knowledge base from which to draw, will benefit more from the open-inquiry environment created by OPBI as opposed to LI or GPBI. This effect was magnified in low-achieving students. This group of students, when instructed by the OPBI method, exhibited improved content acquisition and understanding (MC exam questions) and vastly improved critical reasoning skills compared to both GPBI and LI groups (Figure A1.3C). These results indicate that typically low-achieving students are stimulated by the self-directed and collaborative nature of OPBI, and subsequently are able to achieve a greater level of conceptual understanding of complex physiological subject matter.

There are five main characteristics of OPBI that may have facilitated enhanced performance in typically low-achieving students (Figure A1.6). The first is a collaborative, peer learning environment that encourages students from all academic backgrounds to work together, and has been shown to increase student success in secondary and undergraduate STEM education (Lumpe and Staver 1995, Springer, Stanne et al. 1999). Secondly, this method of learning includes an inherent sense of individual responsibility and self-directed learning that not only garners improved course performance but also establishes student confidence for success when they enter the professional environment. One of the biggest obstacles with these learners is motivating them in the classroom.
OPBI provides an environment that motivates these students in both the traditional way (grades) as well as by creating a demand for knowledge (need to know) and holding them responsible to their peers. Next, low-achieving students benefit from the clearly-defined learning goals that are intrinsic of well-designed constructivist methods (Barrows 1996). Finally, cooperative formative assessments and instructional scaffolding allows for early identification of problem areas in low-achieving students and provides a preexisting toolset to address these areas.

The collaborative environment created by OPBI is essential in regard to elevating the learning strength of low-achieving students. When students work together, the individual intellectual burden of learning can be diminished by exploiting the unique skill and knowledge sets of individual group members (Pea 1993, Salomon 1993, Hmelo-Silver 2004). This is especially true when low and high achieving students are working together (Gabriele and Montecinos 2001). The relationship between student and instructor is a critical aspect of collaboration within groups, and each has a specific role to play to best utilize the group dynamic. The instructor’s role is to ensure that all students are making active contributions to their group and intervening with leading questions when needed (Mayo, Donnelly et al. 1995, Maudsley 1999). Students have many responsibilities to the group. They must contribute meaningfully to the group discussion, not only by presenting their own ideas, but they must also listen to the ideas of other group members and adjust their opinion and process of
thinking when necessary. Students’ duties extend beyond the classroom, in order to effectively contribute to the team, students must come to class prepared to discuss the day’s topics (Donnelly and Fitzmaurice 2005).

The concept of extended learning beyond the classroom strongly promotes individual responsibility and the development of self-directed and self-regulated learning skills. As educators, we have some control over how students develop learning strategies and habits while they are in the classroom, but outside of the classroom they must be responsible for their own learning. Several studies have investigated the ability of students of various achievement levels to develop self-directed learning skills, and found that low-achieving, undergraduate students struggle to develop these skills (Albaili 1997, Ruban and Reis 2006). The development of external study and learning skills could be one of the key disadvantages separating low achieving students from their average- and high-achieving counterparts. There is substantial evidence, both qualitative and quantitative, that supports the effectiveness of PBL at developing self-directed learning skills (Blumberg 2000, Hmelo and Lin 2000, Sungur and Tekkaya 2006, Loyens, Magda et al. 2008). The results of this study suggest that the performance of low-achieving students instructed by the OPBI method is elevated to that of average-achieving students, who out-perform low-achieving students instructed by LI methods (Figure A1.5 & A1.6). This may be due in part, to enriched self-directed learning skills in low-achieving students in the OPBL group. Interactions with other traditional higher achieving students likely
contributed significantly to assisting the lower achieving students in improved learning skills. These aspects of how OPBI improves learning in this student group are currently being explored.

In order to achieve these first two objectives, the course must create a motivating learning environment that garners interest in subject matter. It has been shown that low-achieving students’ performance is enhanced when interest in the subject matter is high (Belloni and Jongsma 1978). In addition to traditional motivations such as passing examinations and earning high course grades, OPBI approaches this obstacle from a couple angles. First, the problems and tasks required of students during class periods are intrinsically motivating by creating a need to know and curiosity in the topics that persist over time (de Volder, Schmidt et al. 1986, Hidi and Renninger 2006, Rotgans and Schmidt 2011). Secondly, the group dynamic motivates the individuals to come to class prepared to discuss the day’s material so that they can make a meaningful contribution, thereby stimulating interest, self-directed learning and a more productive classroom environment. Lastly, clearly defined learning goals (i.e. hypothesis generation, data analysis and critical thinking skills) help to keep low-achieving students on task, interested and motivated to learn (Fuchs, Fuchs et al. 1997, Gabriele and Montecinos 2001).

Formative assessment and instructional scaffolding are critical to ensure student success in problem based learning environments, and this is especially true in
low-achieving students (Barron, Schwartz et al. 1998). The effectiveness and benefits of scaffolding in constructivist methods has been eloquently reviewed (Hmelo-Silver, Duncan et al. 2007). Formative assessment is an essential tool for instructors to identify conceptual areas of need, and in open problem-based instruction this is incorporated into daily classroom activities of small groups and formal class discussions. To maximize the effect of these tools it is imperative to design them to work together in a dynamic way, so that scaffolding is responsive to formative assessment. In conclusion, these data demonstrate that low-achieving students benefit from the motivating, simulating and supportive nature of open problem-based instruction. This instructional method promotes success in this population of students. Future research on this instructional method is designed to help elucidate the potential mechanisms by which lower achieving students improve performance from open problem-based instruction.
Figure A1.6: Key characteristics that make OPBL such an effective tool.
A1.6 ACKNOWLEDGEMENTS

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Ca2+-induced regulation of ion channel and MAP kinase functions." Nature 376(6543): 737.


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Tao, R., C.-P. Lau, H.-F. Tse and G.-R. Li (2008). Regulation of cell proliferation by intermediate-conductance Ca2+-activated potassium and volume-sensitive chloride channels in mouse mesenchymal stem cells.


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2. Franklin BM, Maroudas E, Osborn JL. Sine-wave electrical stimulation initiates a voltage-gated potassium channel-dependent soft tissue response characterized by induction of hemocyte recruitment and collagen deposition. Physiol Rep, 4 (12), 2016, e12832, DOI: 10.14814/phy2.12832


4. Franklin BM and Osborn JL. The Calcium-Activated Chloride Channel, Anoctamin-1, Regulates Cellular Proliferation via Erk1/2 Phosphorylation and Calcium Signaling. AJP: Cell Physiology. (In Review)
Abstracts


7. **Brandon M. Franklin**, S. Randal Voss and Jeffrey L. Osborn. The Calcium-Activated Chloride Channel, Anoctamin-1, is a Critical Regulator of Proliferation in Amphibian Tail Regeneration and This Effect is Mediated via p44/42 MAPK Phosphorylation. *FASEB Journal 29, 2015.*


