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Irreversible Zinc Block of the Swelling-activated Chloride Current in DI TNC1 Astrocytes

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IRREVERSIBLE ZINC BLOCK OF THE SWELLING-ACTIVATED CHLORIDE CURRENT IN DI TNC1 ASTROCYTES

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in Physiology & Biophysics at Virginia Commonwealth University

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ABBREVIATIONS

5-HD……....5-Hydroxydecanoate

Ach……..Acetylcholine

ATP……. Adenosine triphosphate

C1C…… Muscle chloride channel

CFTR……... Cystic Fibrosis Transmembrane receptor

CHF……... Congestive Heart Failure

DCPIB…….. Dichloroxybutiric acid

DI TNC1……Diencephalic astrocyte, *rattus norvegicus* cell line

Diaz……... Diazoxide

DMEM……Dulbecco's Modified Eagle Medium

Ebs…… Ebselen

E_{Cl}…… Chloride equilibrium potential

EGF/R……. Endothelin growth factor receptor

HEPES…….. 4-(2-Butyl-6,7-dichlor-2-cyclopentyl)-indan-1-on-5-yl

Cs_2EGTAH_2…….. Cesium ethylene glycol tetra-acetic acid

IC_{50} ……...Concentration at which 50% inhibition is achieved

$I_{Cl,swell}$…….. Swelling activated chloride current

K_{ATP}……Mitochondrial potassium channel

Mg-ATP……... Magnesium adenosine triphosphate

MTS ……...Methanethiosulfonate

MTSEA-biotin -…….. Methanethiosulfonate ethylammonium with biotin tag

MTSES -……...2-Sulfonatoethyl methanethiosulfonate sodium salt

NADPH……...Nicotinamide adenine dinucleotide phosphate-oxidase

NEM…… N-ethylmaleimide

NMDG……. N-methyl-D-glucamine

NOX …….. NADPH oxidase

PI-3K…….. Phosphoinositide-3-kinase

pICln……... protein proposed to be responsible for $I_{Cl,swell}$

ROS……... Reactive oxygen species

RVD …… Regulatory volume decrease

SITS…… 4-acetamido-4'-isothiocyanato-stilbene-2,2'-disulfonic acid

SWELL1……..… component believed responsible for ICl,swell; also known as LRCC8A

TBI ……Traumatic brain injury

Tris-GTP ……Guanosine-5'-triphosphate

Trypsin-EDTA …….Trypsin Ethylenediaminetetraacetic acid

VRAC ……...Volume regulated anion channel

VSOAC.……..Volume sensitive osmolyte anion channel

VSOR……. Volume sensitive outwardly rectifying channel

ABSTRACT

IRREVERSIBLE ZINC BLOCK OF THE SWELLING-ACTIVATED CHLORIDE CURRENT IN DI TNC1 ASTROCYTES

By: Noah Belkhayat
A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science at Virginia Commonwealth University

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The swelling-activated chloride current, commonly referred to as $I_{Cl,swell}$, is an outwardly-rectifying anion current that plays an important role in cell volume regulation, among other capacities. Despite several decades of research, the molecular identity of the channel responsible for this chloride current remains controversial. Recent indications that key endogenous sulfhydryl groups are capable of modifying the current led us to assess the effects of several divalent cations, including zinc, on $I_{Cl,swell}$. Zinc is known to tightly associate with sulfhydryl groups such as in zinc finger proteins. We found that extracellular zinc irreversibly inhibited $I_{Cl,swell}$ at a site downstream in the signaling cascade. Moreover, zinc blocking kinetics were voltage dependent, suggesting interaction with a site within the

electric field, across the pore of the channel responsible for $I_{Cl,swell}$. The importance of sulfhydryl groups was confirmed by demonstrating irreversible block by N-ethylmaleimide, a sulfhydryl alkylating reagent. In contrast, nickel failed to block $I_{Cl,swell}$, and as noted in previous studies, cadmium preferentially blocked the time-dependent component of $I_{Cl,swell}$. These data confirm the importance of sulfhydryl groups in the function of $I_{Cl,swell}$. Moreover, by demonstrating the voltage-dependence of block, the data strongly suggest the critical sulfhydryl group is within the channel pore. These biophysical characteristics of native $I_{Cl,swell}$ are markers that should be recapitulated in expressed proteins claimed to be responsible for $I_{Cl,swell}$.

CHAPTER 1: INTRODUCTION

The volume regulated anion channel (VRAC), the volume sensitive outwardly rectifying anion channel (VSOR), and the volume sensitive organic osmolyte-anion channel (VSOAC) are polynyms for the ubiquitously expressed anion channel present in mammalian tissues that mediates the current $I_{Cl,swell}$. This channel and perhaps others are responsible for volume regulation of cells in the face of an osmotic gradient. In addition to volume regulation, VRAC has been putatively linked to membrane potential regulation, cell proliferation, and apoptosis (Mongin, 2016). The current passed by VRAC is known as $I_{Cl,swell}$ with an anion permeability sequence of $I^->SCN^->NO_{3-}$->Br->Cl->aspartate>gluconate (Cahalan et al., 1988). Despite recent efforts at further characterization, the molecular identity of VRAC remains controversial.

Although $I_{Cl,swell}$ can be recorded in the absence of extacellular and intracellular calcium, the current is modulated by other divalent cations. Work by Ren and Baumgarten (2005) found that cadmium (Cd^{2+}) suppresses the time-dependent inactivation of $I_{Cl,swell}$ that appears at positive potentials in cardiomyocytes. Furthermore, recent work with murine DI TNC1 astrocytes in our lab suggests that sulfhydryl groups are critical for the function of native $I_{Cl,swell}$ (Park, 2016).

The divalent cation zinc is commonly associated with cysteine residues in cellular proteins and can be chelated by sulfhydryl groups, such as in zinc finger proteins (Pace and

Weerapana, 2014; Noh et al., 2015). Therefore, we postulated that zinc might inhibit $I_{Cl,swell}$ by interaction with sulfhydryl moieties associated with cysteine residues.

1.1 Significance and history of $I_{Cl,swell}$

1.1.1 Significance

VRAC has been found in numerous mammalian cell types, including but not limited to human embryonic kidney cells, human gastrointestinal cells, human T lymphocytes, murine astrocytes, and cardiomyocytes (Cahalan & Lewis, 1988; Hazama & Okada, 1988; Kimelberg, 2005). It is activated in response to swelling, and is key to maintaining cell volume homeostasis. In all cell types, regulating cell volume is critical to survival. Osmotically induced cell swelling can lead to cell lysis. Excessive cell shrinkage leads to protein misfolding and, ultimately, apoptosis.

In traumatic brain injury, damage to the blood brain barrier enables cytotoxic edema which leads to astrocyte swelling. $I_{Cl,swell}$ activation has been linked to this swelling in an attempt to mediate regulatory volume decrease in vivo (Haskew et al., 2002). However, malfunction of $I_{Cl,swell}$ has also shown to contribute to cytotoxicity supporting efflux of both glutamate and aspartate (Haskew et al., 2002).

Work on cardiac myocytes confirms $I_{Cl,swell}$ has multiple functions. This current contributes to cell volume regulation, cell membrane potential, action potential duration, the response to stretch, and is activated in models of heart failure (Clemo & Baumgarten, 1999; Ren & Baumgarten, 2005). In addition, it is known to protect against ischemia-reperfusion injury (Mulvaney et al., 2000).

1.1.2 History

Using whole cell patch clamping, Cahalan and Lewis (1988) first observed an outwardly rectifying chloride current, with an E_{rev} = -45 mV, in murine T lymphocytes. Their studies revealed a chloride current with apparent ATP dependence as well as an osmotic gradient dependence, and most intriguingly a membrane stretch dependence in order to be activated. Simultaneously, Hazama and Okada (1988) determined that human epithelial cells exhibited a regulatory volume decrease (RVD) when hypotonically challenged that was mediated by a chloride current. Decreasing extracellular chloride concentration in conjunction with hypotonic challenge facilitated the regulatory volume decrease. They found that chloride channel blockers, specifically SITS, inhibits the observed regulatory volume decrease. Both groups are credited with discovering $I_{Cl,swell}$; however it was not referred to by this name until the mid-1990's.

1.1.3 Attempts at molecular characterization

Claims that candidates such as P-glycoprotein (Valverde et al., 1992; Gill et al. 1992) and regulator pICln (Paulmichl et al. 1992), as well as specific members of the C1C family of channels (Duan et al., 1999), are responsible for $I_{Cl,swell}$ have been firmly rejected by numerous groups working to identify VRAC. More recent claims regarding the molecular identity for VRAC have also been controversial. The claim that SWELL1 (LRRC8A), a leucine-rich transmembrane protein, is an essential component of VRAC was proposed in 2014 (Qiu et al., 2014; Voss et al., 2014). Using siRNA techniques to knockdown LRRC8A, significantly decreased VRAC currents were reported in Xenopus Oocytes, Human Embryonic Kidney Cells, HeLA cells, and CD4+ T lymphocytes. However, others demonstrated $I_{Cl,swell}$ activation in the absence of LRRC8A in Human

Embryonic Kidney cells (HEK293), HeLa cells, and HCT116 cells and concluded that this protein is not essential (Sirianant et al., 2016). One of the problems groups face when working on $I_{Cl,swell}$ is the lack of a high-affinity ligand, which has limited efforts to directly purify the channel protein.

1.2 Biophysical properties of $I_{Cl,swell}$

1.2.1 Rectification and time dependent inactivation

Under physiological chloride conditions, where $[Cl^-]_I$ is 20 mM and [Cl-]o is 100 mM, $I_{Cl,swell}$ undergoes outward rectification with a reversal potential near that of E_{Cl} which is -42 mV (Cahalan et al., 1988). Under symmetrical chloride conditions, $I_{Cl,swell}$ still maintains outward rectification at a modified reversal potential of 0 mV. In contrast, other chloride currents, such as $I_{Cl,cAMP}$ do not rectify in symmetrical chloride (Ren & Baumgarten, 2005). As a result, this characteristic has been used to identify $I_{Cl,swell}$. Additionally $I_{Cl,swell}$ is a calcium-independent chloride channel, and can be elicited in the absence of extracellular calcium combined with strong intracellular calcium buffering as previously indicated (Hagiwara et al., 1992; Tseng, 1992).

Interestingly, $I_{Cl,swell}$ maintains a time dependent component exhibited in multiple cell types. At highly positive potentials, $I_{Cl,swell}$ exhibits time-dependent decay in rabbit cardiomyocytes (Ren & Baumgarten, 2005), murine astrocytes (Park, 2016), as well as other cell lines. The basis for this time dependent decay is unknown. Cadmium (Cd^{2+}), added to the bath solution, is able to negate the decay up to at least +100 mV and reveals a slow delayed rectifying current that is blocked by addition of 1 mM barium (Ba^{2+}). This

combination of divalent cations facilitates a time-independent expression of $I_{Cl,swell}$ at various potentials (Ren & Baumgarten, 2005).

1.2.2 Permeability sequence and conductance

Eisenman produced a series of 11 sequences for both monovalent cations and anions that proposed ion selectivity is based on the energy required to remove an ion from its hydration shell and the electrostatic energy required for binding of the ion to a site in the channel (Hille, 2001). VRAC has a permeability sequence that obeys the Eisenmann type I selectivity sequence for anions: I^->SCN^->NO_{3-}>Br->Cl->aspartate>gluconate (Mongin, 2016). In this sequence, the rate-limiting step is believed to be the dehydration of anions.

The average single channel conductance for VRAC is 50-80 pS at positive potentials, and 10-20 pS at negative potentials (Mongin, 2016). This conductance is much higher than members of the ClC family of chloride channels, as well as the CFTR family, which are largely considered to have conductance values in the single pS range.

1.2.3 Pathways of activation.

$I_{Cl,swell}$ can be activated by multiple stimuli that act through a complex cascade. Under iso-osmotic conditions, $I_{Cl,swell}$ has been activated by cell inflation (Hagiwara et al., 1992), anionic amphipaths (Tseng, 1992), β1-integrin stretch receptor activation (Browe & Baumgarten, 2003), AngII (Browe & Baumgarten, 2004), EGF (Browe & Baumgarten, 2006), H_2O_2 a ROS derivative (Browe & Baumgarten, 2004), and ET-1 (Du & Sorota, 2000; Deng et al., 2009). In vivo activation of $I_{Cl,swell}$ is largely attributed to hypotonic challenge ultimately leading to reactive oxygen species (ROS) production or ROS production under isosmotic conditions.

1.2.3 (a) Hypotonic Challenge

$I_{Cl,swell}$ is known to activate as a result of hypotonic challenge which induces cell swelling. Cell swelling itself is non-specific with regards to activation of $I_{Cl,swell}$ as it can activate a membrane stretch response, reduce ionic strength, dilute ion concentration, or alter macromolecule concentration. All of which have the potential to act as a volume sensor in the cascade of $I_{Cl,swell}$ activation (Browe and Baumgarten, 2003).

$I_{Cl,swell}$ activation via membrane stretch has been elucidated in work with cardiomyocytes by Browe and Baumgarten (2003). Activation of β-1 integrin stretch receptors triggers a signaling cascade with Angiotensin II as an intermediate that ultimately elicits $I_{Cl,swell}$ via interaction with NADPH oxidase (NOX) as seen in Figure 1. A selective blocker of $I_{Cl,swell}$, tamoxifen, blocks the identified current in the presence of stretch.

$I_{Cl,swell}$ activation mediated by swelling is also due to upstream AT_1 receptor activation by Angiotensin II which proceeds to activate EGFR kinase and upregulate PI-3 kinase activity resulting in ROS production by NOX. It appears that mechanical membrane stretch and osmotic swelling, both, stimulate many of the same signaling molecules that lead to current activation.

1.2.3 (b) Activation induced by ROS

As downstream effectors in the stretch and swelling-activated pathway, reactive oxygen species (ROS) are known activators of $I_{Cl,swell}$. Both NADPH oxidase and the mitochondria are two sources of ROS known to activate $I_{Cl,swell}$ (Browe and Baumgarten, 2003; Deng et al. 2009). NADPH oxidase, a distal component of the stretch activated pathway, is activated by EGF/R and PI-3K following stimulation by Angiotensin II. NADPH oxidase activation results in production of reactive oxygen species (ROS). This

ROS subsequently interacts with the mitochondria resulting in further ROS production including O_2^- which is rapidly dismutated to a membrane permeant H_2O_2 as can be seen in Figure 1. This final form of ROS is what ultimately activates $I_{Cl,swell}$ with its site of action hypothesized to be at the channel responsible for the chloride current, however that remains unconfirmed. Browe and Baumgarten (2003) show extracellular catalase is capable of inhibiting $I_{Cl,swell}$ lending credence to this hypothesis.

Additionally mitochondrial ROS, in the absence of hypotonic challenge and downstream of NADPH oxidase activation, can act to elicit $I_{Cl,swell}$ as well. Application of 20 μM acetylcholine (Ach) elicits a Cl^- current with characteristics of $I_{Cl,swell}$ that is blocked by 5HD, a mitochondrial K_{ATP} channel inhibitor (Browe & Baumgarten, 2005). Furthermore the complex III inhibitor and K_{ATP} channel opener diazoxide also activates a Cl^- current with the same biophysical and pharmacological characteristics as $I_{Cl,swell}$ under isosmotic conditions (Browe & Baumgarten, 2005).

Separately, application of exogenous H_2O_2 has also been shown to elicit $I_{Cl,swell}$, which is subsequently quelled by extracellular catalase (Browe and Baumgarten 2003). The ability to quench ROS using antioxidants, either endogenous or exogenous, has proven critical to modulating $I_{Cl,swell}$.

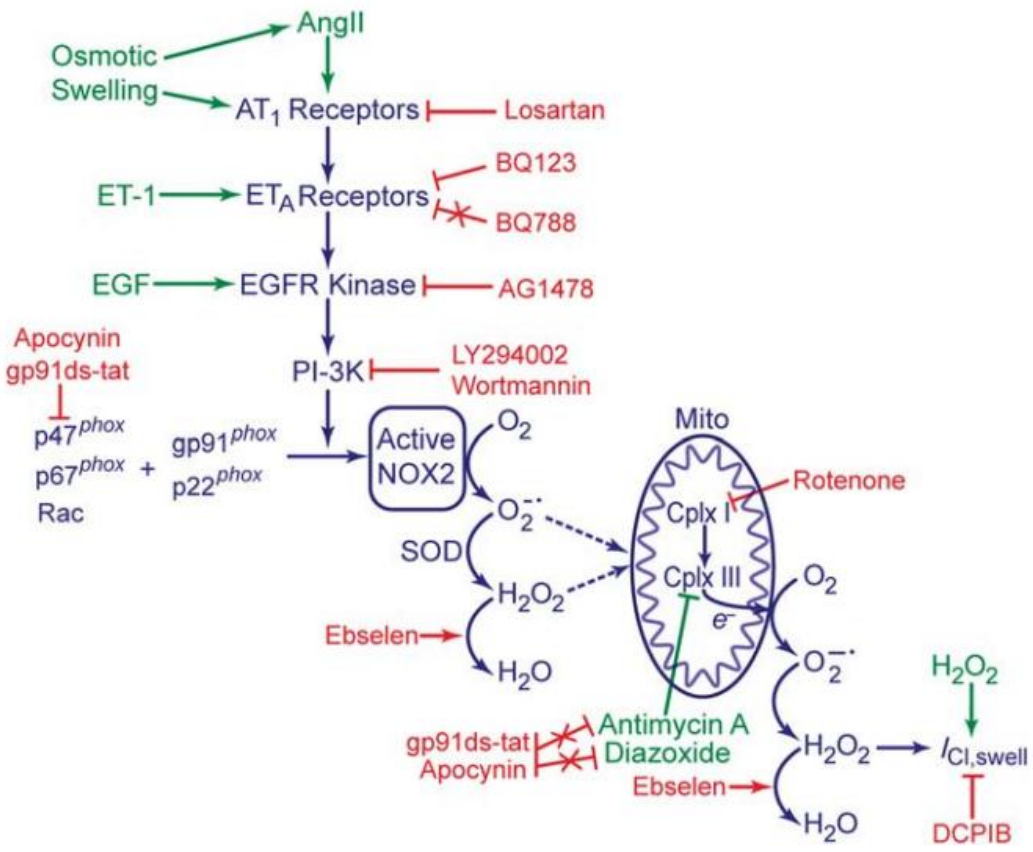

Figure 1: Signaling cascade involved in activating $I_{Cl,swell}$ elucidated by Deng et al. (2009). Both NADPH oxidase (NOX) and the mitochondria produce ROS that subsequently activate $I_{Cl,swell}$. In this situation, ebselen is believed to be acting as a peroxidase, dismutating intracellular H_2O_2 to H_2O, quenching ROS. Application of exogenous H2O2 alone is capable of eliciting $I_{Cl,swell}$.

1.3 Pharmacology of $I_{Cl,swell}$

Stated earlier, a major factor contributing to the difficulty in identifying the channel responsible for $I_{Cl,swell}$ is finding a high affinity, and highly selective, blocker of the Cl^- current. To date, the most potent blocker of $I_{Cl,swell}$ is an ethacrynic-acid derivative known as DCPIB (Decher et al., 2001). The IC_{50} of DCPIB for $I_{Cl,swell}$ is 2.7 μM (Liang et al., 2014), and 10 μM DCPIB is routinely used to reversibly inhibit the Cl^- current in pulmonary artery smooth muscle cells, Xenopus oocytes, guinea pig atrial cardiomyocytes, and murine diencephalic astrocytes. Its selectivity is believed superior when compared to the estrogen receptor antagonist tamoxifen, as DCPIB does not inhibit I_{Na}, I_{Ca}, or I_{C1C}, and I_{Ks} (Decher et al., 2001).

An earlier agent, DIDS, is another molecule capable of inhibiting $I_{Cl,swell}$. Like Dideoxyforskolin and verapamil, DIDS is capable of distinguishing between $I_{Cl,swell}$ and CFTR. However these agents, along with many others, appear tissue specific with regards to their efficacy (Decher et al., 2001).

Recent work by Park (2016) has shown that sulfhydryl modifiers, specifically charged MTS reagents, are capable of inhibiting $I_{Cl,swell}$ in different cell types such as DI TNC1 astrocytes and HEK-293 human embryonic kidney cells. Charged MTS reagents, specifically MTSES and MTSEA-biotin, are alkylthiosulfonates capable of forming disulfide linkages with sulfhydryl groups of cysteine residues (Kenyon & Bruice, 1977). These findings suggest a critical sulfhydryl group, extracellularly available, is capable of modulating $I_{Cl,swell}$.

Another important molecule capable of modulating $I_{Cl,swell}$ is ebselen, a seleno-organic small molecule with known affinity for sulfhydryl groups. This anti-inflammatory,

cytoprotective compound is a membrane permeant glutathione peroxidase mimetic that dismutates H_2O_2 to H_2O. It has been shown to suppress $I_{Cl,swell}$ in cardiomyocytes by Deng et al. (2009). This action, believed to be occurring intracellularly, distal to NADPH oxidase ROS production, results in the quenching of ROS directly responsible for activating $I_{Cl,swell}$ as can be seen in Figure 1.

1.4 Objective of the present study

New work by Park (2016) has shown that ebselen, capable of abrogating $I_{Cl,swell}$, appears to mediate its actions by sulfhydryl modification. It is well known that ebselen binds metallothioneins, a family of cysteine rich proteins, with high affinity. Its affinity for cysteine residues is attributed to a strong electrophilic potential which mediates the formation of a covalent selenium-sulfur bond between the selenium of ebselen and the sulfur of cysteines (Sakurai et al., 2006). Ebselen-metallothionein interaction has been linked to the oxidation of endogenous thiolate ligands by the selenium of ebselen, which results in the ejection of zinc (Jacob et al., 1998).

Zinc (Zn^{2+}), a trace element with high affinity for thiols in-vivo, has been shown to bind directly to cysteine and histidine residues of certain cation and anion channels, including voltage gated potassium channels and skeletal muscle chloride channels. Zinc has been shown to either augment or inhibit activity of these channels (Duffield et al., 2005; Noh et al., 2015) by modifying gating conditions.

In extending the research into divalent cation modulation of ion channels, this study assesses the following major aims:

1. Determine whether zinc, or other divalent cations, modulate $I_{Cl,swell}$
2. If zinc is found to inhibit $I_{Cl,swell}$, determine if block by zinc is voltage dependent
3. Based on the findings by Park (2016) suggesting sulfhydryl involvement, determine if sulfhydryl modifiers inhibit $I_{Cl,swell}$ downstream of H_2O_2.

This study highlights the novel findings of an irreversible inhibition of $I_{Cl,swell}$ by zinc, acting at a site downstream of H_2O_2, and lends credence to the existence of a sulfhydryl group that is capable of modulating $I_{Cl,swell}$. Furthermore the ability of zinc to block $I_{Cl,swell}$ in a voltage dependent manner indicates zinc senses the electric field, and proposes that zinc has a binding site in the pore of the channel responsible for $I_{Cl,swell}$. In comparing block by zinc to other divalents we find that zinc does not inhibit the decay component of $I_{Cl,swell}$ as cadmium does. In concert with nickel's inability to inhibit $I_{Cl,swell}$, this reveals a distinct mechanism that exists for zinc block of the chloride current. Finally, inhibition of $I_{Cl,swell}$ by the sulfhydryl modifier N-ethylmaleimide provides further evidence of a critical sulfhydryl group downstream of H_2O_2 that is consistent with previous work by Park (2016).

CHAPTER 2: MATERIALS AND METHODS

2.1 Culture of DI TNC1 astrocytes

DI TNC1 astrocytes are an immortalized cell line from one day old rat diencephalon (Radany et al., 1992). Uniquely they do not contain aquaporins, mediating water transport through ion channels including $I_{Cl,swell}$. DI TNC1 astrocytes passages 5-20 were used for these studies. Tissue culture dishes (5 mL x 60 x 15 mm) were pretreated with Corning CELLBIND. DI TNC1 cells were cultured in the pretreated dishes using a solution of DMEM (Corning Cellgro, 10-013-CV) supplemented with 10% by volume fetal bovine serum (Sigma-Aldrich, F2442), and 1% by volume penicillin/streptomycin (Cellgro 30-002-CI). The cells were kept at 37°C in an atmosphere of 5% CO_2 and 95% air at a relative humidity of approximately 95%. Once the cells reached confluence, approximately 70-80% in terms of density, the cells were split and the passage increased by 1. To split DI TNC-1 cells, a brief wash with approximately 5 mL of HyClone (Thermo Scientific, SH40007-13) was followed by incubation with 4 mL of 0.25% trypsyin-EDTA (gibco/Thermo Fisher Scientific, 25200056) for 20 min at 37°C. Once the cells had dissociated from the surface of the dish, 8 mL of DMEM solution was applied to the plate to inhibit trypsin. The total volume, with cells, was transferred to 15 mL centrifuge tubes. The cells were centrifuged at 2500 RPM for 2-4 minutes using an IEC centrifuge device. Following this, the supernatant was removed by aspiration, and the pellet gently re-

suspended in 5 mL of DMEM solution. Immediately following this, 2.0 mL of the cell containing solution was plated onto a plate containing 5 mL of DMEM solution and the passage was increased by 1. This plate was placed back in the incubator. The remaining cells, suspended in 3 mL DMEM in the centrifuge tube, were incubated for 1 h. Following incubation the tube was centrifuged again and the supernatant aspirated. The pellet was re-suspended in 5 mL of 1T bath solution for use when patch clamping.

2.2 Experimental solutions and drugs

Bath and pipette solutions were designed to isolate the Cl^- current. Isosmotic bath solution (1T; 320 mOsm/kg; where T indicates times isosmotic) contained (in mM): 90 N-methyl-D-glucamine-Cl, 3 $MgCl_2$, 10 HEPES, 10 glucose, 5 CsCl, 0.2 $CdCl_2$ and mannitol was added to adjust solution osmolarity to approximately 320 mOsm. Except as noted in Figure 12, cadmium is always included in the bath solution. Solution pH was adjusted to 7.35 using CsOH. Isosmotic pipette solution contained (in mM) 110 NMDG-Glucuronate, 20 TEA-Cl, 0.15 $CaCl_2$, 10 Cs_2EGTAH_2, 10 HEPES, 5 Mg-ATP, and 0.1 Tris-GTP. Additionally 0.053 mM diazoxide, a mitochondrial K_{ATP} channel activator, was added to the pipette solution to ensure $I_{Cl,swell}$ activation. Pipette solution pH was adjusted to 7.2. The bath solution was stored at room temperature and pipette solution was purged under argon gas and frozen at -20°C in 500 µL aliquots.

Diazoxide (Sigma Aldrich, 364-98-7) was prepared at a stock concentration of 40 mM and stored in 500 µM aliquots at -20°C. It was added to pipette solution for a final concentration of 53 µM. DCPIB (Sigma Aldrich, 82749-70-0), an ethacrynic-acid derivative, was prepared as a 10 mM stock solution in 1T and aliquoted into 100 µM

aliquots. Aliquots were stored at -20°C. Stock solution was added to the bath solution immediately before use to reach the desired concentration discussed in the results section.

The stock solution of 100 mM zinc in water was prepared using anhydrous zinc (II) chloride (Sigma Aldrich, 7646-85-7). Zinc chloride was dissolved under heat to avoid formation of zinc hydroxychloride precipitates. The solution pH was neutral and was stored at 2°C. Stock solution was added to 1T bath solution immediately before use to reach the desired concentration discussed in results section. The stock solution of 100 mM nickel in water was prepared using nickel (II) chloride (Sigma Aldrich, 7791-20-0). The solution pH was tested to 4.2 to ensure nickel hydrolysis in solution. Stock solution was stored at 2°C. Stock solution was added to 1T bath solution immediately before use to reach the desired concentration discussed in the results section. N-ethylmaleimide (NEM) (Sigma Aldrich, E3876) was stored at -20°C. The head space of the containing bottle was purged with argon in an attempt to prevent exposure to air. It was prepared in 1T solution at experimental concentrations immediately prior to use. Stock solution of 200 mM cadmium chloride in 1T was kept at 2°C and added into the bath solution at 200 μM for studies, except as indicated. Stock hydrogen peroxide (H_2O_2) 30% by wt in aqueous solution (Fisher Scientific, 7722-84-1) was stored at 2°C. Preparation of 500 μM H_2O_2 in 1T bath solution occurred immediately prior to use to avoid decomposition.

2.3 Whole-cell patch clamp and electrophysiological recordings

Following re-suspension in 5 mL of 1T solution, DI TNC1 cells were pipetted onto the glass bottom of a recording chamber and allowed to settle for 10 minutes. The recording chamber was placed onto the stage of an inverted light microscope (Nikon) with Hoffman

modulation optics. A high resolution video camera (CCD72, Dage-MTI) with projection to an analog television was used to visualize individual cells. Cells were suprafused with bath solution at 2.0-2.3 mL/min and recordings were made at room temperature, approximately 21-23°C. Single use glass pipettes were pulled from 10 cm long, 1.1 mm inner diameter thin-walled borosilicate capillary tubing (Sutter Instruments). Using a Narishige MF-83 polisher, pipettes were fire polished to a final tip diameter of approximately 2.0-2.5 microns with a final resistance, in bath solution, of 3-5 MΩ. These pipettes were back filled with diazoxide–containing pipette solution prior to attachment to the holder, with average time between backfilling and giga seal formation of 4-4.5 min. Membrane currents were recorded in the whole-cell configuration using an Axopatch 200B amplifier. The ground electrode was a 3-M KCl agar bridge connected directly to the bath solution. Seal resistances were between values of 2-10 GΩ prior to breaking into the cell. A quick burst of negative pressure, via manual stimulation, allowed access to the cell or "break-in". Axial resistances after break in were below 10 MΩ. All data was corrected for the measured liquid junction potential.

Voltage clamp protocols were established using the software pClamp 8.2 (Axon Instruments). Consecutive 550 ms voltage steps were implemented from a holding potential of -60 mV. Consecutive test potentials ranged from -100 mV to +80 mV in 10 mV increments. Membrane currents were low-pass filtered at 2 kHz and digitized at 5 kHz. A second protocol was generated with a modified holding potential. This protocol was used in part of the zinc studies. It involves the same test potentials as the aforementioned protocol, but with a modified holding potential of 0 mV. I-V curves are representative of averages across the duration (550 ms) of the voltage step unless otherwise noted in the

results section. To minimize variability, experiments were designed to use cells as their own controls. Control currents were obtained immediately upon break in. Zero time for recordings began as soon as break in occurred. Average time between backfilling pipette with solution and break in was approximately 4-4.5 minutes.

2.4 Intracellular application of agents

Backfilling the pipette with the aforementioned agents is necessary to isolate $I_{Cl,swell}$. Immediately after breaking into the cell, the first current traces are recorded under conditions that mimic basal chloride current expression. Identifying this basal $I_{Cl,swell}$ expression allows us to use each cell as its own control. Dialysis of the pipette solution with cellular contents is diffusion limited, thus there is some small delay before diazoxide induces activation of $I_{Cl,swell}$. The subsequent activation of the chloride current can then be compared to the initial conditions and allows us to determine control values for a given experiment without having to gather excess data from separate cells.

2.5 Statistics

Statistics were generated using SigmaStat, the statistical component of SigmaPlot 12.5. Patch clamp current density data (pA/pF) are reported as mean ± SEM, and n is the number of cells. Reporting data in terms of current densities and as fractional current accounts for differences in astrocyte membrane surface area and expression of the channel. For multiple comparisons between different treatments, a One-Way Repeated measures analysis of variance (ANOVA) was performed followed by a Holm-Sidak test for pair wise comparison. In all cases, $P<0.05$ was considered to be significant.

CHAPTER 3: RESULTS

3.1 Identification of $I_{Cl,swell}$ in DI TNC1 astrocytes with DCPIB

In addition to several other biophysical characteristics, identification of $I_{Cl,swell}$ in mammalian tissues is achieved pharmacologically by using the molecule DCPIB (Decher et al., 2001). Several studies demonstrate that DCPIB is a high affinity, selective blocker of $I_{Cl,swell}$ as administration of DCPIB fails to inhibit other anion currents, including those elicited by CFTR, Ca-activated Cl^- channels, members of the ClC family as well as several cation currents, specifically I_{Na}, I_K, and I_{Ca} (Decher et al., 2001). The selective and reversible block by DCPIB makes it a useful tool for identification of $I_{Cl,swell}$ (*cf.*, Deng et al., 2016; Bowens et al., 2013). Thus to confirm we were observing $I_{Cl,swell}$, we added DCPIB to the bath solution and used bath and pipette solutions designed to isolate anion currents.

$I_{Cl,swell}$ is activated under isosmotic conditions by diazoxide (Diaz), a mitochondrial K_{ATP} channel activator that causes the release of ROS from complex III (Deng et al., 2009). In the present studies, we evoked $I_{Cl,swell}$ by backfilling the patch pipette with diazoxide (Diaz [in]; 53 μM) immediately before breaking into DI TNC1 astrocytes. Figure 2 illustrates families of currents, corresponding I-V relationships, fractional currents, and the time course of current at +60 mV. Following break-in, $I_{Cl,swell}$ slowly turned on and reached a steady-state. As expected for $I_{Cl,swell}$, the current outwardly

rectified and reversed near the Cl^- equilibrium potential (E_{Cl}), -42 mV in physiological Cl^- solutions. To pharmacologically identify $I_{Cl,swell}$, DCPIB (10 μM) was added to the bath solution. DCPIB inhibited diazoxide-induced $I_{Cl,swell}$ by 93.2 ± 7.6% (n = 4, P <0.001) at +60 mV in 10.6 ± 1.3 min. In three of these experiments, reversibility of block of $I_{Cl,swell}$ by DCPIB was confirmed. Washout of drug in isosmotic (1T) bath solution restored $I_{Cl,swell}$ to 73.3 ± 7.6% (n =3, P<0.01) of fully activated $I_{Cl,swell}$. Note that block by DCPIB reduced the anion current to a value less than that of the first recording after break-in (Fig. 2D; open circle, dashed line). This suggests that a fraction of $I_{Cl,swell}$ was already activated at the initial time point. Some delay between breaking-in and the initial recording is unavoidable because the instrumentation settings must be adjusted. Therefore, diazoxide may have begun to act before the recording was made. Moreover, partial activation of $I_{Cl,swell}$ is sometimes observed and blocked by DCPIB under isosmotic conditions in the absence of a stimulus to elicit the current.

Another characteristic of $I_{Cl,swell}$ that distinguishes this anion channel from others is that $I_{Cl,swell}$ rectifies in symmetrical Cl^- solutions (Baumgarten et al., Mechanosensitivity in Cells and Tissues, 2005). Although we did not confirm this biophysical characteristic in DI TNC1 cells, recent studies in DI TNC1 cells by Park (2016) confirmed the outward rectification under symmetrical chloride conditions. Taken together, outward rectification in physiologic and symmetrical Cl- gradients and block by DCPIB meet the diagnostic criteria to verify the current studied in DI TNC1 astrocytes is $I_{Cl,swell}$.

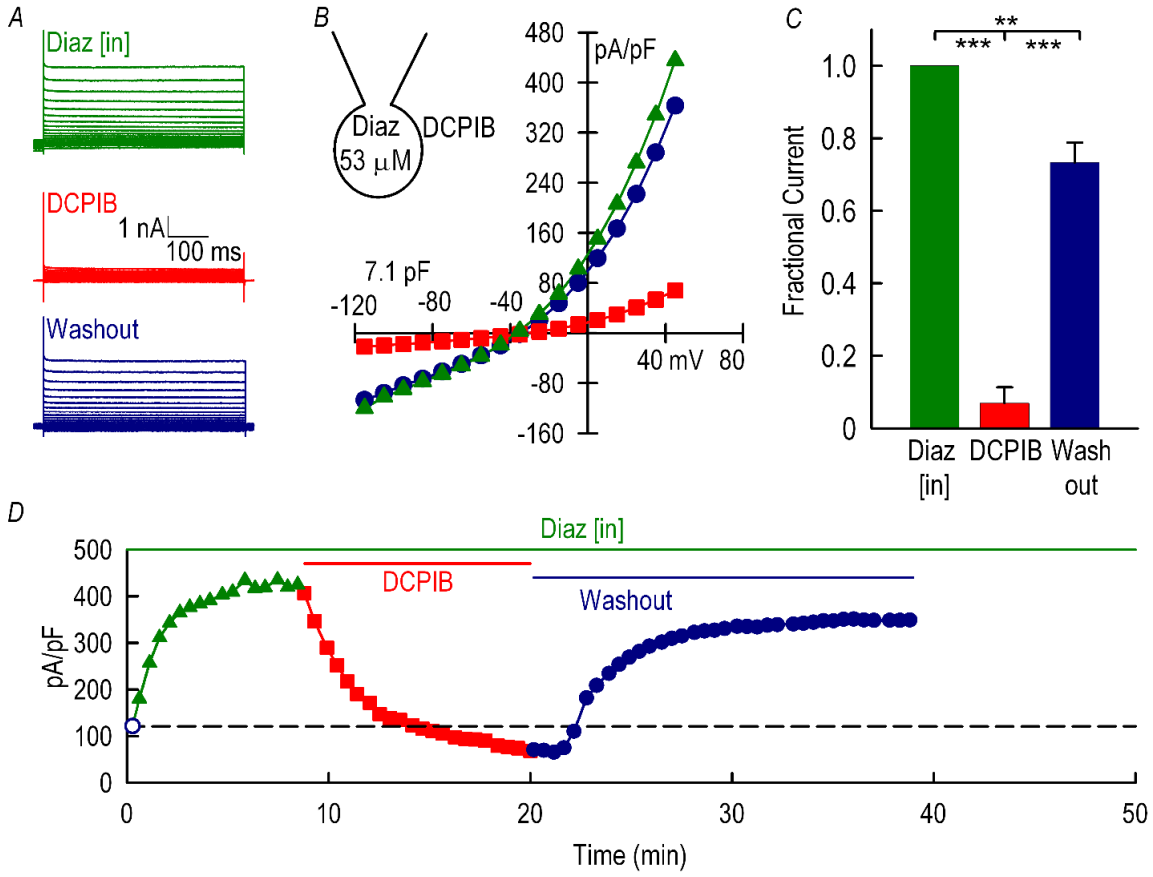

Figure 2: Extracellular [10 μM] DCPIB inhibited $I_{Cl,swell}$ and was reversible, with DCPIB washout restoring diazoxide-induced $I_{Cl,swell}$. (***A***) Families of currents at full activation of diazoxide-induced $I_{Cl,swell}$ (Diaz [in]), and after block by DCPIB, and after DCPIB washout. (***B***) Current-Voltage (I-V) relationships for A. Cell capacitance 7.1 pF. (***C***) Fractional currents recorded after initial full activation of diazoxide-induced $I_{Cl,swell}$ (Diaz [in]), after DCPIB mediated inhibition of $I_{Cl,swell,}$ and after washout of DCPIB. DCPIB inhibited 93.2 ± 7.6% (n =4, P<.001) of diazoxide induced $I_{Cl,swell}$ in 10.6 ± 1.3 min. Washout of DCPIB restored $I_{Cl,swell}$ to 73.3 ± 7.56% (n =3, P<.01) of steady state current diazoxide induced current. (***D***) Time course indicating break in (○), activation induced by diazoxide (▲), block by DCPIB (■) and DCPIB washout (●).

3.2 $I_{Cl,swell}$ is maintained upon activation by diazoxide in DI TNC1 astrocytes

To eliminate the possibility that activation of $I_{Cl,swell}$ was only transient and that current might spontaneously run down, we tested whether current activation was maintained. As shown in Figure 3, inclusion of diazoxide (53 μM) in the patch pipette under isosmotic conditions activated $I_{Cl,swell}$, and current reached a steady-state value in 15.3 ± 2.1 min (n = 3). Moreover, current activation was maintained for the duration of the recording, 50.3 ± 13.1 min (n = 3), without evidence of rundown. The time to activation was based on repeated pulses to +60 mV yielding the same current values (Fig. 3D; arrow) and maintained current taken from the last pulse (arrow). Rather than running down, the maintained current was slightly, but not significantly, greater than the initial steady state current (Fig. 3C). This is consistent with unpublished recordings in DI TNC1 cells by Park (2016) showing diazoxide-induced $I_{Cl,swell}$ remains turned on for more than 5 hours without run down. These studies served as an important control for time and established that inclusion of diazoxide in the patch pipette caused prolonged activation of $I_{Cl,swell}$. Therefore, block of diazoxide-induced $I_{Cl,swell}$ by subsequent addition of other agents cannot be attributed to rundown.

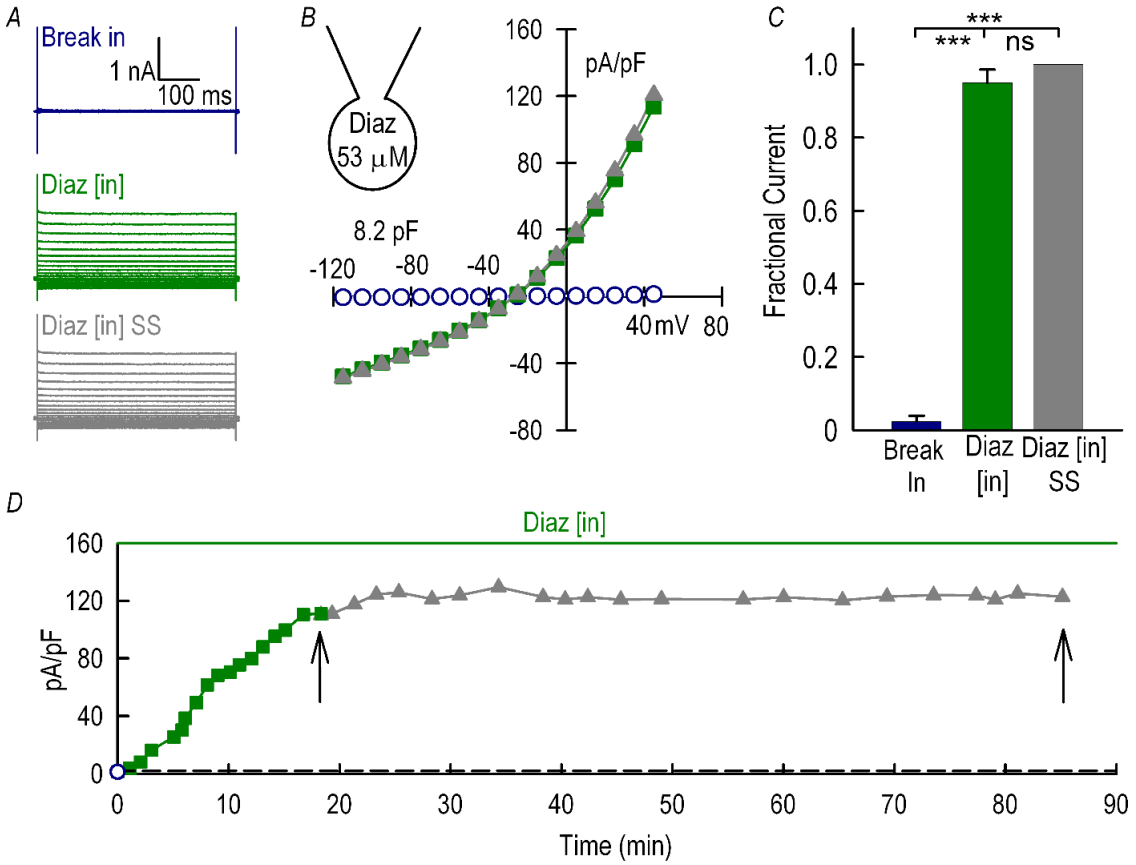

Figure 3: Diazoxide-induced I*Cl,swell* does not run down during prolonged diazoxide exposure. (***A***) Families of currents, (***B***) I-V relationships and (***C***) fractional currents recorded at break in with [53 μM] diazoxide in patch pipette (Break in), after initial full activation (Diaz [in] Init) at 15.3 ± 2.1 min after break in, and in steady state (Diaz [in] SS) at 50.3 ± 13.1 min after initial full activation. Initial and steady state diazoxide-induced $I_{Cl,swell}$ were not significantly different (n = 3, P = 0.445); currents normalized by current in steady state. (***D***) Time course indicating break in (○), initial activation by diazoxide (■) and maintained diazoxide-induced $I_{Cl,swell}$ (▲); times of initial (Init) and steady state (SS) recordings are indicated as above. Arrows (↑) correspond to points at which families of currents for initial full activation (Diaz [in]) and steady state (Diaz [in] SS) were obtained.

3.3 Inhibition of $I_{Cl,swell}$ in DI TNC1 astrocytes mediated by the divalent Zn^{2+}

Recent studies in the laboratory by Park (2016) suggest the involvement of a critical sulfhydryl group capable of modulating $I_{Cl,swell}$ in DI TNC1 astrocytes. These studies indicate that charged, membrane impermeable MTS reagents added to the bathing media block $I_{Cl,swell}$. This strongly suggests that one or more sulfhydryl groups, accessible from the extracellular face of the membrane, are critical to channel function. In addition ebselen, a potent glutathione peroxidase mimetic, is known to abrogate ICl,swell. It has been shown that ebselen is capable of interacting with sulfhydryls of cysteine groups and releasing coordinated zinc from those sites (Jacob et al., 1998).

These findings lead us to hypothesize that the divalent cation zinc would inhibit $I_{Cl,swell}$, an anion channel. Based on the chemistry of zinc and experimental evidence, a number of biological actions of zinc are attributed to its chelation by protein sulfhydryl groups (Noh et al., 2015). To test this hypothesis, $I_{Cl,swell}$ was activated by inclusion of diazoxide in the patch pipette, and after stable activation of the current was achieved, zinc was added extracellularly as a component of the flowing bath solution.

3.3.1 Extracellular zinc inhibited $I_{Cl,swell}$

Figure 4 illustrates the effect of zinc (100 μM) applied extracellularly after pipette diazoxide caused steady-state activation of $I_{Cl,swell}$. Zinc inhibited $I_{Cl,swell}$ by 92.3 ± 1.6% (n = 4, P <0.001) after 44.9 ± 3.0 min (n = 4) in zinc-containing bathing solution. After allowing for the solution change, inhibition of $I_{Cl,swell}$ by zinc was well described by a single exponential with a time constant, τ, of 18.8 ± 2.1 min (n = 4).

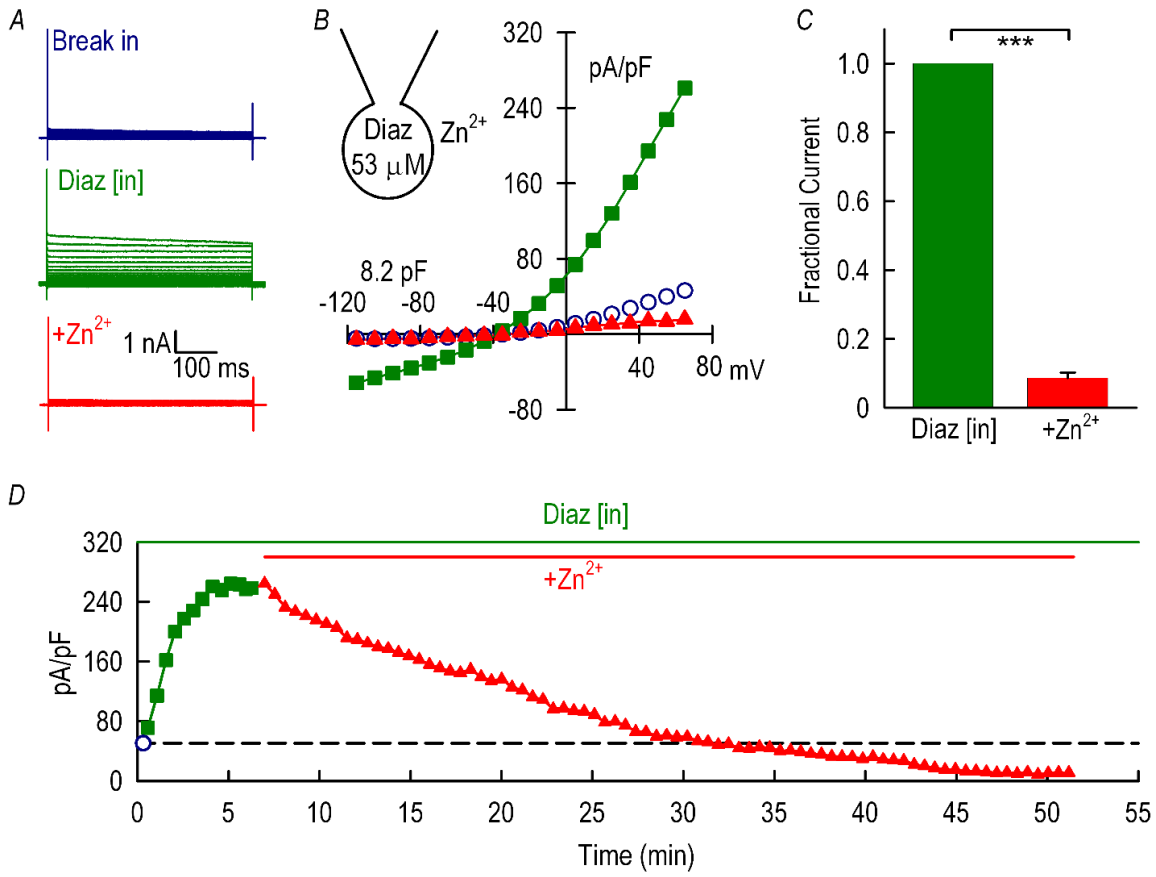

Figure 4: **Extracellular zinc (100 µM) inhibited $I_{Cl,swell}$.** (***A***) Families of currents including current inhibited by extracellular zinc. (***B***) Corresponding I-V relationships and (***C***) fractional currents recorded at steady state activation of diazoxide-induced $I_{Cl,swell}$ and at zinc block normalized to steady state current. Zinc inhibited 92.3 ± 1.6% (n =4, P<.001) of steady state current in 44.9 ± 3.0 min (n =4). (***D***) Time course indicating break in (○), activation induced by diazoxide (■), and inhibition by zinc (▲).

This kinetic model implies that the rate of inhibition of $I_{Cl,swell}$ by zinc ($1/\tau$) should be proportional to the concentration of zinc in solution. Figure 5 shows that increasing the zinc concentration 3-fold, to 300 μM, inhibited diazoxide-induced $I_{Cl,swell}$ more rapidly than 100 μM zinc, as expected. Full block occurred in 15.3 ± 0.4 min (n =10) with a time constant, τ, of 6.4 ± 0.9 min (n = 10). Comparing the time constants, the rate of inhibition of $I_{Cl,swell}$ by 300 μM zinc was 2.94-times faster than by 100 μM zinc. The approximately 3-fold decrease in the observed time constant for inhibition was commensurate with the 3-fold increase in concentration of zinc.

Preliminary experiments showed 50 μM zinc was an effective blocker and inhibited 97.7 ± 2.7% of $I_{Cl,swell}$ but block took 88 ± 7.9 min to develop (n = 2; data not shown). The average time constant was 39.4 ± 2.1 min (n = 2). Time to full block by 50 μM zinc was about twice that for 100 μM zinc, as expected. Because the slow onset of block at 50 μM zinc required prolonged recordings that were difficult to obtain, further kinetic analysis at this concentration was not undertaken.

3.3.2 Block of $I_{Cl,swell}$ by zinc was irreversible

To test whether the effect of zinc on diazoxide-induced $I_{Cl,swell}$ was reversible, zinc (300 μM) was washed out of the bath solution after inhibition of $I_{Cl,swell}$. $I_{Cl,swell}$ failed to significantly recover during prolonged wash out, 43.1 ± 8.7 min, in zinc-free bath solution (Fig. 5C-D, n = 4). These findings suggest that block of $I_{Cl,swell}$ was due to a tight interaction between zinc and its target. This stands in contrast to block of $I_{Cl,swell}$ by DCPIB, which is rapidly reversible upon removal of drug from the bath solution. With regards to reaction kinetics, the lack of recovery of $I_{Cl,swell}$ after zinc washout implies that the off-rate constant for zinc at its binding site is negligible when compared to the on-rate

constant, and significant dissociation of zinc is unlikely to occur within the time frame of a patch clamp experiment.

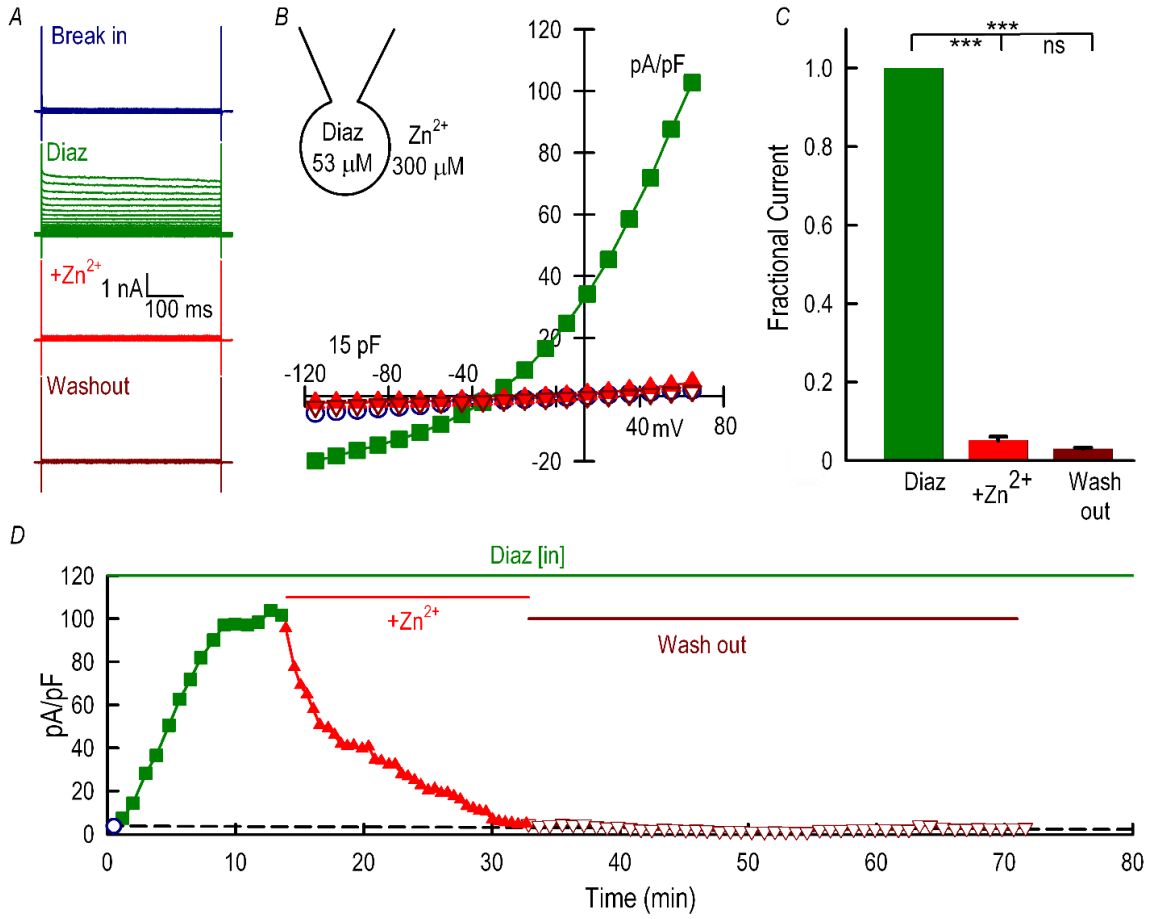

Figure 5: [300 µM] zinc inhibited $I_{Cl,swell}$, and was irrecoverable with zinc washout. (***A***) Families of currents, (***B***) I-V relationships and (***C***) fractional currents recorded at steady state diazoxide-induced $I_{Cl,swell}$, zinc block, and washout. Fractional current normalized to steady state $I_{Cl,swell}$. Zinc inhibited 94.2 ± 2.3% (n =10, P<.001) of steady state diazoxide-induced $I_{Cl,swell}$ in 15.3 ± 0.4 min (n = 10). Inhibition was maintained during washout with 3.0 ± 0.2% (n =4, P<.001) of steady state current observed over a time period of 43.1 ± 8.7 min (n = 4). (***D***) Time course indicating break in (○), activation by diazoxide to steady state $I_{Cl,swell}$ (■) , inhibition by zinc (▲), and washout (▽).

3.3.3 Kinetic Analysis of Zinc Mediated Inhibition

The kinetics of ion channel inhibition by a drug often can be described as a pseudo first order reaction with 1:1 binding of a drug, D, to a fixed number of channels, *C, as for example, the block of voltage-gated sodium channels by 9-aminoacridine (9-AA) and of potassium channels by TEA (Khodakhah et al., 1997).

$$*C + D \underset{k_b}{\overset{k_r}{\leftrightarrows}} *CD$$

This results in current inhibition that fits a single exponential function. The fractional change in $I_{Cl,swell}$ current over time (-dI/dt) is proportional to the fraction of channels that conduct, C,

(1) $-dI/dt = \{k_b [D] C\} - \{k_r (1 - C)\}$

and the time constant of inhibition, τ, is given by

(2) $\tau^{-1} = k_b [D] + k_r$

where k_b is the rate constant for block, k_r is the rate constant for unblock, and [D] is the concentration of drug, in this case zinc. Because block by zinc appeared to be irreversible, k_r must be negligible compared to k_b [D], and Equ. 1 and 2 can be simplified to

(3) $-dI/dt = \{k_b [D] C\}$

(4) $\tau^{-1} = k_b [D]$

Block of $I_{Cl,swell}$ by both 100 μM and 300 μM zinc was well described by single exponential functions. The reaction rates, τ^{-1}, were 0.053 ± 0.008 μM·min^{-1} for 100 μM zinc (n =4) and 0.157 ± 0.006 μM·min^{-1} for 300 μM zinc (n =10). This approximately 3-fold difference in reaction rate is consistent with the 3-fold increase in zinc concentration.

3.3.4 Zinc inhibits $I_{Cl,swell}$ at a downstream site

The experiments described so far do not shed light on the location of zinc block. Diazoxide is thought to activate $I_{Cl,swell}$ via mitochondrial ROS production (see Figure 1). One might imagine, for example, that zinc interferes with mitochondrial ROS production, acts at a regulatory site downstream from cellular ROS production but upstream from the channel, or binds to the channel itself. Studies characterizing the signaling cascade responsible for eliciting $I_{Cl,swell}$ found that exogenous H_2O_2 retains its ability to turn on the current when upstream signaling is blocked and is the most downstream activator identified to date (Browe and Baumgarten, 2003,2004; Deng et al., 2009). Therefore, we tested whether block by zinc was upstream or downstream to the site of activation of $I_{Cl,swell}$ by exogenous H_2O_2.

Figure 6 shows activation of $I_{Cl,swell}$ by diazoxide, block by 300 μM zinc, brief washout (5 min) to clear zinc from the flowing bath solution, and the response to 500 μM H_2O_2. Zinc inhibited 93.1 ± 3.2 % (n = 6, P <0.001) of the diazoxide-induced $I_{Cl,swell}$, and no recovery of $I_{Cl,swell}$ was observed during washout. Importantly, prolonged exposure to H_2O_2 in bath solution, 52.2 ± 22.1 min (n = 6), failed to significantly reactivate $I_{Cl,swell}$ after inhibition by zinc. This strongly suggests that the site of current block by zinc is downstream to that of current activation by exogenous H_2O_2. The time course illustrated in Fig 6D shows the longest treatment with H_2O_2, almost 120 min. This recording was unusual, however, in that a small fraction of $I_{Cl,swell}$ that was inhibited by zinc appeared to recover within about 5 min. The basis for this modest recovery of current in the presence of H_2O_2 was unclear.

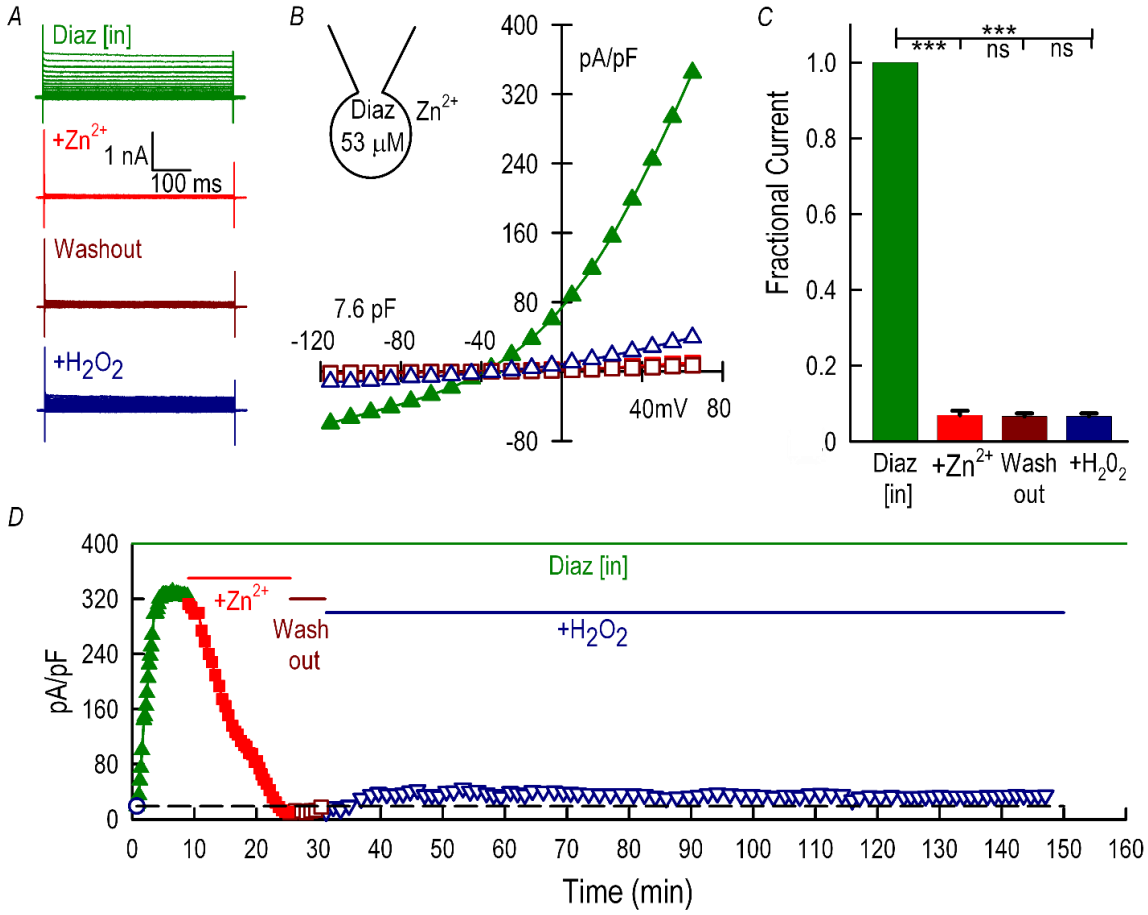

Figure 6: Extracellular 300 µM zinc inhibited $I_{Cl,swell}$, which was not recoverable in the presence of exogenous 500 µM H_2O_2. (***A***) Families of currents including currents during treatment with H_2O_2. (***B***) Corresponding I-V relationships and (***C***) fractional currents recorded normalized to steady state diazoxide induced $I_{Cl,swell}$. Zinc inhibited 93.5 ± 3.4 % (n =6, P<.001) of diazoxide-induced $I_{Cl,swell}$. H_2O_2 failed to recover 92.9 ± 0.8% (n =6, P<.001) of diazoxide-induced $I_{Cl,swell}$ over an average time period of 52.2 ± 22.1 min. The above time course shows exposure to H_2O_2 for 118.6 min. (***D***) Time course indicating break in (○), activation induced by diazoxide (▲), block by zinc (■) washout (□) and exposure to H_2O_2 (▽).

3.4 Voltage-dependence of zinc block of $I_{Cl,swell}$

Finding that block by zinc was downstream to $I_{Cl,swell}$ activation by H_2O_2 raised the possibility that zinc directly blocked the channel. For example, several cations inhibit cation channels by binding within the channel pore, and block is voltage-dependent because the binding site is within the electric field (Steinbach 1968; Adams 1977; Neher et al. 1978). Might zinc block $I_{Cl,swell}$ in an analogous manner? To test this possibility, we examined whether the kinetics of inhibition of $I_{Cl,swell}$ by zinc was voltage-dependent. As zinc is a divalent cation, if it must enter the electric field in order to block $I_{Cl,swell}$, the kinetics of block should be slower at more positive potentials than at more negative potentials (Hille, 2001; Sheets & Hanck, 1992; Li & Baumgarten, 2001)

The kinetics of block of $I_{Cl,swell}$ by 300 μM zinc with a holding potential of -60 mV was characterized previously (see Fig. 5). Therefore, as illustrated in Figure 7, we repeated the experiment using an identical series of test potentials, except the holding potential was shifted to 0 mV. With a holding potential of 0 mV, the time constant, τ, for block was 31.7 ± 1.8 min, yielding a reaction rate of 0.032 ± 0.003 $\mu M \cdot min^{-1}$ (n = 4). In contrast, at a holding potential of -60 mV, the time constant, τ, for block was 6.4 ± 0.9 min (n = 10), and the rate of block was 0.157 ± 0.005 $\mu M \cdot min^{-1}$ (n = 8). Thus, shifting the holding potential 60 mV positive slowed the development of block 5-fold.

This difference in the kinetics of development of block at the two holding potentials strongly suggests that zinc senses the electric field. The fraction, δ, of the electric field (i.e., applied transmembrane voltage) sensed at the zinc binding site can be estimated using Eyring rate theory (Hille, 2001; Gutfreund 1995). This considers the effect of the field on the barrier height, and therefore, the rate constant for hopping over the barrier to bind.

Because the reaction rate is the product of the intrinsic on-rate constant and the apparent concentration of zinc (see Eq. 1), an equivalent result can be obtained using the Nernst equation to estimate the effect of the field in terms on the concentration of zinc at the binding site. Application of the Nernst equation in this situation is given below:

$$(5) \qquad [Zn^{2+}]_{0\ mV}/[Zn^{2+}]_{-60\ mV} = \exp(-zFE\delta/RT)$$

where $[Zn^{2+}]$ is the effective concentration of zinc at 0 and -60 mV, z is its valence, F is Faraday's constant, E is the transmembrane voltage, R is the gas constant, and T is temperature in Kelvin. The reaction rate for block by zinc was 4.9-fold greater at -60 than 0 mV (Figs. 5 and 7), which would result if the concentration of zinc was 4.9-fold greater and rate constant was unchanged (Equ. 4). Solving for the fraction of the field that zinc senses yields δ=0.31. That is to say, zinc must move through 31% of the electric field to reach its binding site.

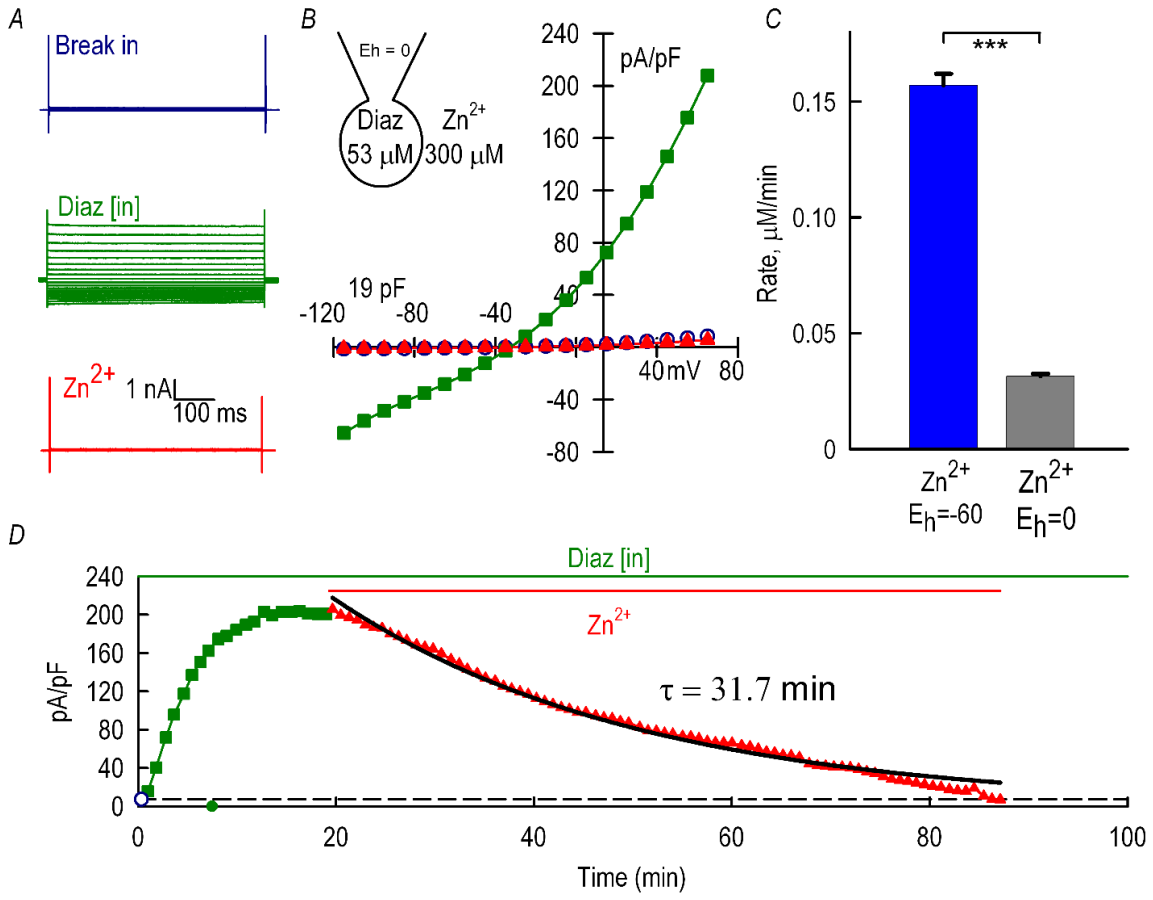

Figure 7: [300 µM] zinc inhibited $I_{Cl,swell}$ at Eh=0 mV with a decreased reaction rate as expected. (***A***) Families of currents, and (***B***) their respective I-V relationships. (***C***) Reaction rates for [300 µM] at a holding potential Eh=-60 mV and Eh= 0 mV. The average rate of reaction for zinc at Eh= -60 mV was 0.157 ± .005 µM.min^{-1} (n=8). The average rate of reaction for zinc at Eh= 0 mV was 0.032 ± .003 µM.min^{-1} (n=4). (***D***) Time course showing break in (○), activation by diazoxide to steady state $I_{Cl,swell}$ (■), and inhibition by zinc (▲). Average time constant, or mean lifetime, was 31.7 ±1.8 min (n=4).

3.5 N-Ethylmaleimide (NEM) irreversibly inhibits $I_{Cl,swell}$ at a downstream site

As previously mentioned, data from our laboratory indicates that $I_{Cl,swell}$ can be inhibited by sulfhydryl modifying agents, including MTS reagents. N-Ethylmaleimide (NEM) is a known Michael acceptor in the Michael Addition reaction, with ability to react with thiol groups generating an irreversible thioether under physiological conditions (Smyth et al., 1960). Thus we hypothesized that extracellular NEM would inhibit $I_{Cl,swell}$ providing further evidence that critical sulfhydryl group(s) modulate $I_{Cl,swell}$.

As illustrated in Figure 8, addition of 400 μM NEM to the bath solution inhibited 94.0 ± 0.9% of diazoxide-induced $I_{Cl,swell}$ in 16.7 ± 1.4 min (n = 7, P <0.001). Subsequent washout of NEM for 18.3 ± 2.1 min failed reactivate diazoxide-induced $I_{Cl,swell}$ and did not produce a significant recovery of current after NEM block (n=4, ns) Thus, the action of NEM is consistent with the idea that irreversible modification of critical sulfhydryl group(s) suppresses channel function.

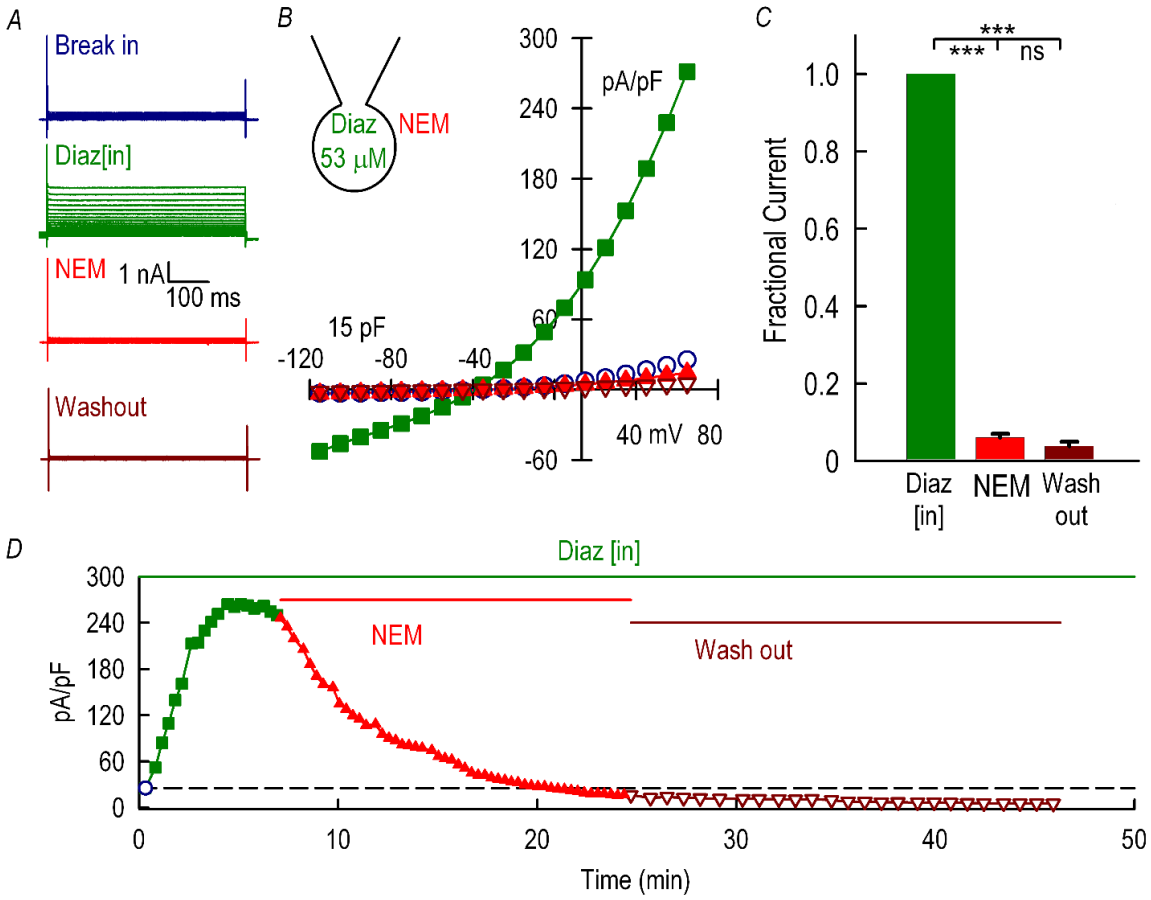

Figure 8: $I_{Cl,swell}$ is irrecoverable after treatment with [400 µM] NEM and subsequent washout. (***A***) Families of currents and (B) corresponding I-V relationships. (C) Fractional currents normalized to steady state diazoxide induced $I_{Cl,swell}$. Average value for steady state diazoxide-induced $I_{Cl,swell}$ was 273.1 ± 64.7 pA/pF (n=7). NEM inhibited 94.0 ± 0.9 % of steady state diazoxide induced $I_{Cl,swell}$ (n=7, P<.001). Washout failed to recover 94.2 ± 1.6 % of $I_{Cl,swell}$. (D) Time course showing break in (○), activation induced by diazoxide (■), inhibition by NEM (▲) in 16.7 ± 1.4 min (n=7), and washout (▽) for 18.3 ± 2.1 min.

3.5.1 H_2O_2 Fails to Elicit $I_{Cl,swell}$

In similar fashion to the aforementioned zinc studies, we wished to elucidate whether the site of action of NEM was upstream or downstream to $I_{Cl,swell}$ activation by H_2O_2. To determine this, we added 500 μM H_2O_2 to the bath solution following a washout of NEM. As illustrated in Figure 9, exposure to H_2O_2 for 45 – 121 min failed to significantly recover $I_{Cl,swell}$ (n = 3) after NEM inhibited 93.6 ± 1.2% of diazoxide-induced current (n = 3, P <0.001). At the end of treatment with H_2O_2, 95.2 ± 1.9% of diazoxide-induced current remained inhibited. The inability of H_2O_2 to elicit $I_{Cl,swell}$ suggests a binding site for NEM downstream of H_2O_2 activity.

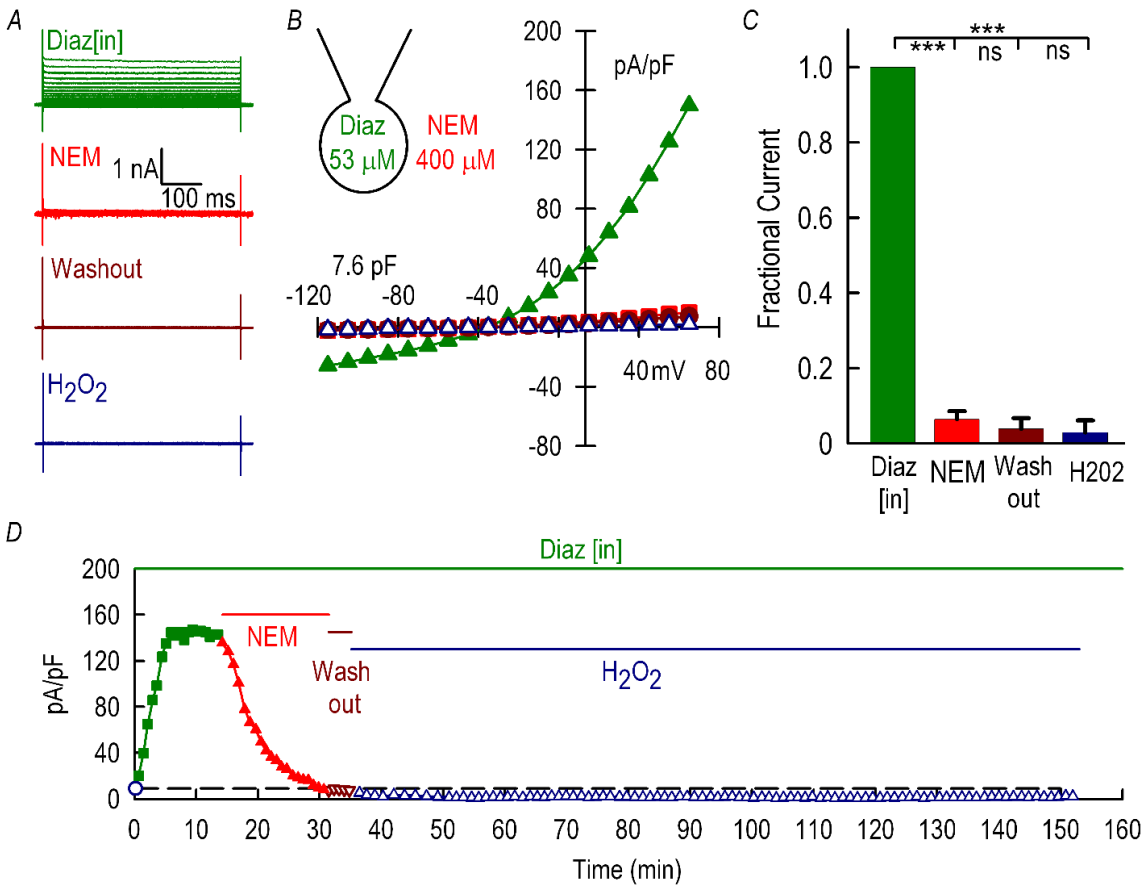

Figure 9: Inability to recover $I_{Cl,swell}$ in the presence of H_2O_2 after NEM washout. (***A***) Families of currents, and (***B***) corresponding I-V relationships. (***C***) Fractional currents normalized to steady state diazoxide-induced $I_{Cl,swell}$. NEM inhibited 93.6 ± 1.2% of diazoxide-induced $I_{Cl,swell}$ (n=3, P<.001). Washout failed to recover 94.3 ± 0.8% of steady state current. Treatment with H_2O_2 elicited 2.9 ± 1.9% of steady state diazoxide-induced $I_{Cl,swell}$. (***D***) Time course showing break in (○), activation by diazoxide to steady state $I_{Cl,swell}$ (■), inhibition by NEM (▲), washout of [400 µM] NEM for 8.2 ± 1.3 min (n=3) (▽), and treatment with [500 µM] H_2O_2 for 75.7 ± 23 min (n=3) (Δ).

3.6 Is there a common mechanism of $I_{Cl,swell}$ inhibition by divalent cations?

To evaluate the possibility of a common mechanism of $I_{Cl,swell}$ inhibition mediated by divalent cations, we tested the response to nickel (Ni^{2+}) under the same conditions employed during experiments with zinc. As previously discussed, nickel in solution has affinity for both cysteines and histidines at physiological pH, albeit with a greater affinity for histidines. Based on the ability of zinc to inhibit $I_{Cl,swell}$, we hypothesized that a similarly sized divalent known to interact with cysteines might also be able to inhibit $I_{Cl,swell}$. Preliminary studies were conducted using 100 and 300 μM nickel, and a more definitive analysis was done using 1 mM nickel.

3.6.1 Prolonged exposure to 100 μM nickel failed to inhibit $I_{Cl,swell}$

In a similar fashion to zinc, we applied 100 μM nickel to the bath solution once diazoxide-induced $I_{Cl,swell}$ had reached steady state. Figure 10C shows a time course of a preliminary experiment in which application of 100 μM nickel for 30.4 ± 4.7 min (n = 2) failed to inhibit diazoxide-induced $I_{Cl,swell}$. $I_{Cl,swell}$ in the presence of exogenous nickel was 98.6 ± 1.7% (n = 2) of steady state diazoxide-induced $I_{Cl,swell}$ prior to nickel treatment.

3.6.2 Exposure to 300 μM nickel failed to inhibit $I_{Cl,swell}$

Increasing the concentration of nickel by 3-fold to 300 μM failed to inhibit diazoxide-induced $I_{Cl,swell}$ over the same time frame as 300 μM zinc. 300 μM zinc was the working concentration, with an average time to full inhibition of $I_{Cl,swell}$ of approximately 15 min. Administration of 300 μM nickel over a time period of 15.1 ± 3.3 min (n = 2) failed to inhibit 98.3 ± 2.1% (n = 2) of diazoxide-induced $I_{Cl,swell}$.

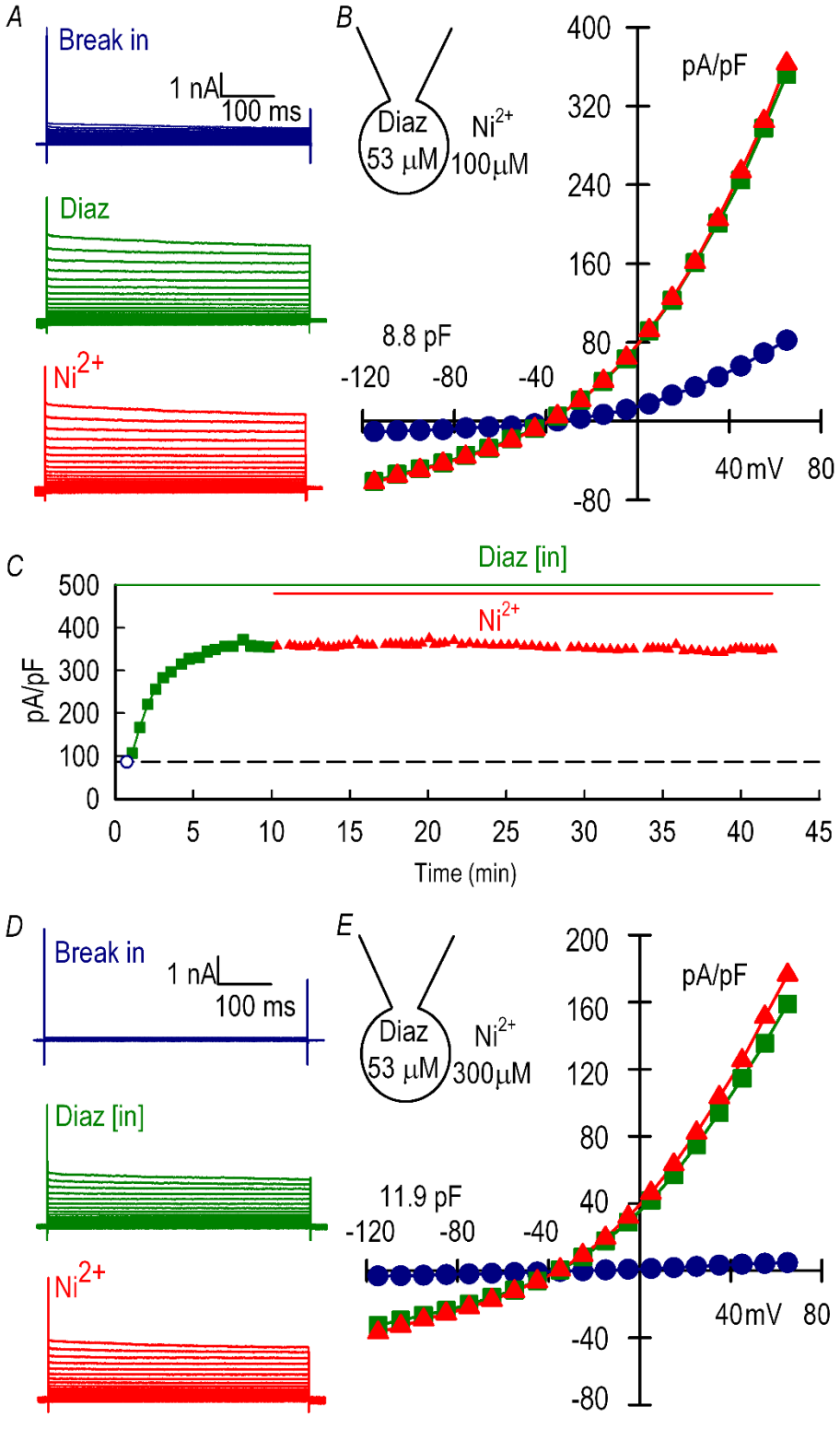

Figure 10: Please see next page for figure legend and description

Figure 10: Extracellular 100 μM and 300 μM nickel failed to inhibit $I_{Cl,swell}$. (***A***) Families of currents for 100 μM nickel, (***B)*** corresponding I-V relationships, and (***C***) a time course indicating 100 μM nickel failed to inhibit 98.6 ± 1.7% (n=2) of diazoxide-induced $I_{Cl,swell}$ over a time period of 30.4 ± 4.7 minutes. (***D***) Families of currents for 300 μM nickel, and (***E***) corresponding I-V relationships with nickel I-V curve (▲) showing a slight increase in $I_{Cl,swell}$ after treatment with [300 μM] nickel for 15.1 ± 3.3 minutes (n=2).

3.6.3 Prolonged exposure to 1 mM nickel failed to inhibit $I_{Cl,swell}$

With no evidence of inhibition at 100 and 300 μM nickel, we increased the concentration of nickel to 1 mM. In these experiments, diazoxide-induced $I_{Cl,swell}$ was 213.9 ± 42.1 pA/pF at steady state (n = 11). Figure 11 shows that even at 1 mM, nickel failed block $I_{Cl,swell}$. Rather, at the end of nickel exposure for 22.3 ± 2.5 min (10.3 to 35.8 min), $I_{Cl,swell}$ was 112 ± 13% of the apparent steady-state diazoxide-induced $I_{Cl,swell}$ (n = 11, P = 0.368). The lack of efficacy of nickel at 1 mM despite full block at much lower concentrations by zinc suggests specific interactions with particular divalents are required to inhibit $I_{Cl,swell}$.

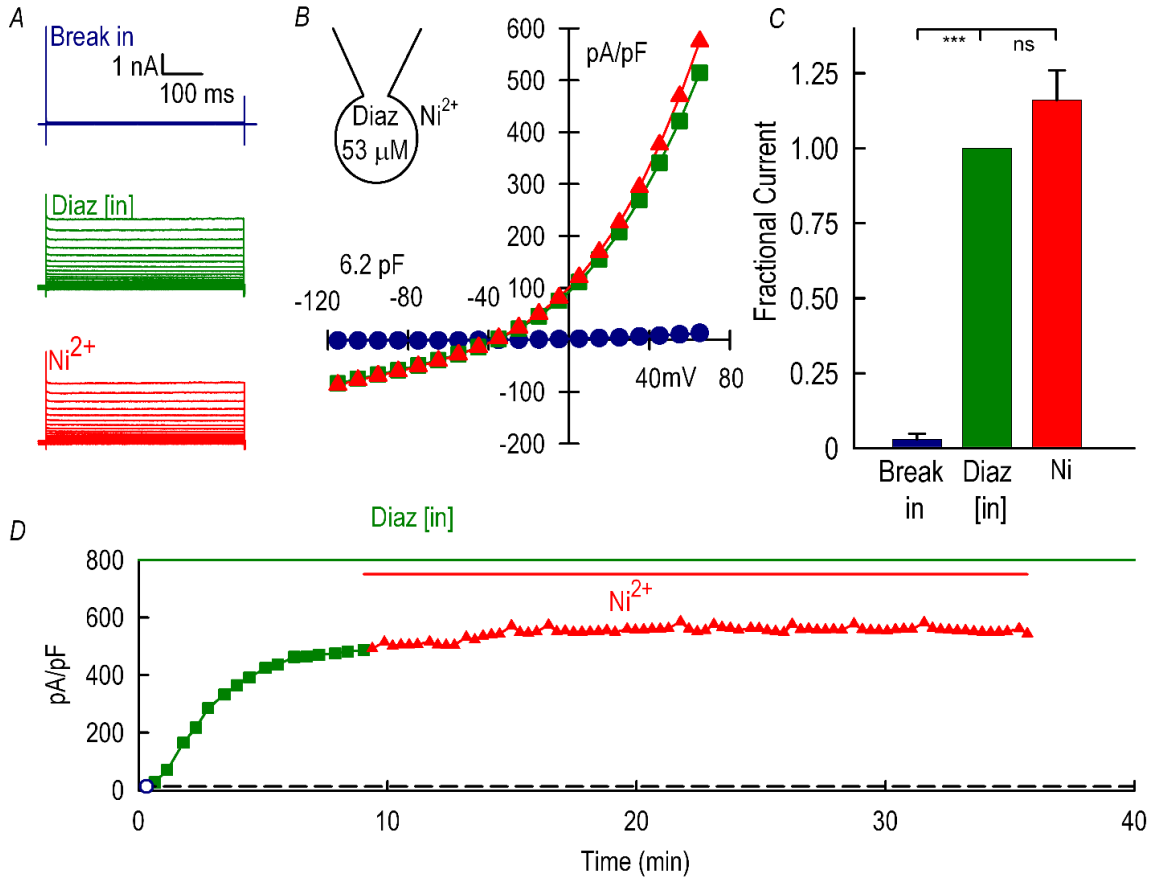

Figure 11: Extracellular 1 mM nickel failed to inhibit $I_{Cl,swell}$ (***A***) Families of currents and (***B***) corresponding I-V relationships. (***C***) Fractional currents recorded normalized to steady state diazoxide-induced $I_{Cl,swell}$. The average current density for diazoxide-induced $I_{Cl,swell}$ was 213.9 ± 42.1 pA/pF (n=11). $I_{Cl,swell}$, in the presence of [1 mM] nickel, increased to 112 ± 13% of diazoxide-induced $I_{Cl,swell}$ (n=11, P=.368). (***D***) Time course indicating break in (○) shortly after time zero, activation induced by diazoxide (■), and treatment with [1 mM] nickel for 22.3 ± 2.5 (n=11) (▲).

3.7 Evaluation of distinct mechanisms for divalent mediated inhibition of $I_{Cl,swell}$

$I_{Cl,swell}$ expressed in cardiomyocytes exhibits a time dependent current decay at strongly positive test potentials (Ren & Baumgarten 2005) and analogous behavior is observed in DI TNC1 astrocytes in our lab. Ren & Baumgarten (2005) showed that addition of 200 μM cadmium (Cd^{2+}) to bath solution suppressed current decay and time-dependence of $I_{Cl,swell}$, but the exact mechanism is unknown. This raised a question, "Is block of $I_{Cl,swell}$ by zinc time-dependent?" To test this, we analyzed the currents inhibited by zinc at different time periods during the 550 ms voltage step.

Figure 12 show 200 μM cadmium eliminated the time dependent inactivation of $I_{Cl,swell}$. Under our standard cadmium-containing bath solution, $I_{Cl,swell}$ was virtually time independent (Fig. 12A). In the absence of cadmium, however, there is a time-dependent decay of $I_{Cl,swell}$ at positive potentials (Fig. 12B). Note that addition of cadmium appeared to suppress the initial decaying current much more strongly than the steady state current.

To test whether 300 μM zinc had differential effects on the initial and steady state current, as shown for cadmium, we compared the currents after approximately 50% block by zinc. As shown in Figure 12C, in the absence of cadmium, zinc blocked both the initial and steady-state components of $I_{Cl,swell}$, and $I_{Cl,swell}$ decayed at positive potentials was observed in the absence of both divalents (Fig. 12A). To quantitatively assess block by zinc during the test pulse, we compared the current at the initial (20-25 ms), early (30-40 ms), and late (490-510 ms) time points during the 550 ms voltage step when zinc had inhibited 33%, 50% or 67% of steady state current. The ratios of early/initial currents and late/initial currents, when compared to steady state diazoxide induced $I_{Cl,swell}$, show no appreciable difference from currents obtained during cadmium-free treatment. This

information suggests there is a distinct mechanism of action between zinc and cadmium. In conjunction with our data that shows nickel did not mediate any significant effect on $I_{Cl,swell}$, these findings argue against a common pathway for divalent mediated inhibition of $I_{Cl,swell}$.

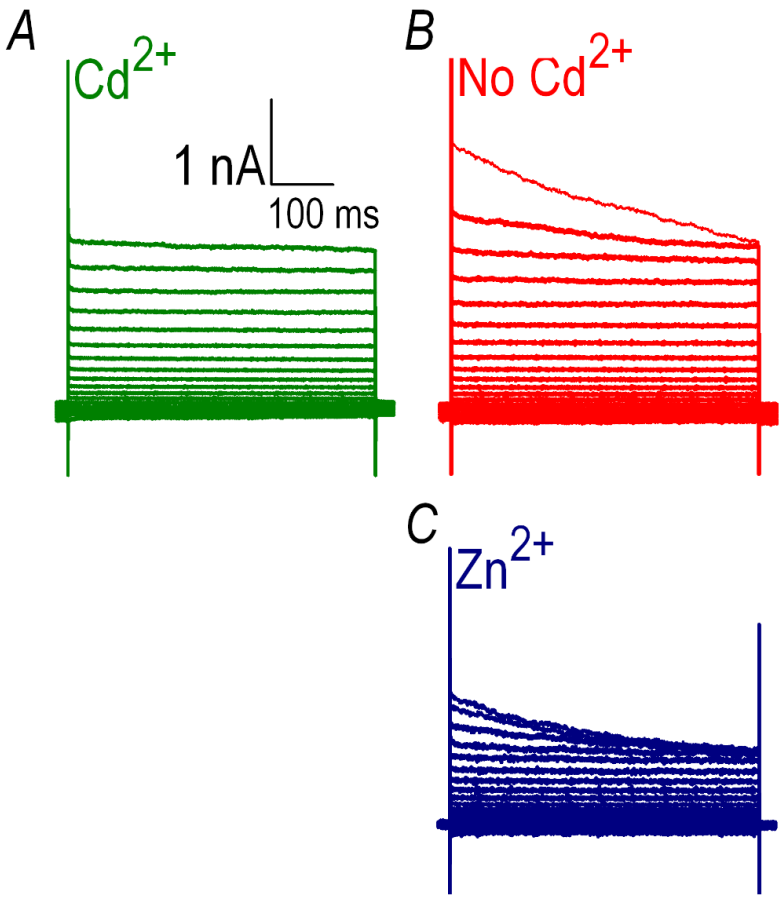

Figure 12: Comparison of block of $I_{Cl,swell}$ by zinc and cadmium. (***A***) Whole cell recording of family of currents at steady state diazoxide-induced $I_{Cl,swell}$ with 200 μM cadmium (Cd^{2+}) in bath solution (standard bath solution). No significant time-dependent current delay was seen at positive test potentials. (***B***) Distinct cell recording of family of currents representing $I_{Cl,swell}$ in the absence of cadmium. Slow decay of current was observed at positive potentials. (***C***) Separate cell with recordings in the absence of cadmium. Both initial and steady state currents were reduced by 300 μM zinc after ~50% block, and time-dependent decay of current at positive potentials was observed. Zinc and cadmium appear to act by different mechanisms.

CHAPTER 4: DISCUSSION

This study demonstrated, for the first time, that the divalent cation zinc is capable of irreversibly inhibiting a Cl^- conductance with the biophysical and pharmacological properties of $I_{Cl,swell}$ in DI TNC1 astrocytes. Furthermore, the rate of current inhibition by zinc was voltage dependent. The simplest explanation for this is that the zinc binding site is within the pore of the channel responsible for $I_{Cl,swell}$ and senses the electric field across the pore. The ability of extracellular N-Ethylmaleimide (NEM) to irreversibly inhibit $I_{Cl,swell}$ lends credence to the theory that a critical sulfhydryl group is responsible for modulating this chloride current. In contrast to zinc, the divalent cation nickel failed to inhibit $I_{Cl,swell}$ and cadmium preferentially blocked the time-dependent component of the current at positive potentials.

4.1 Inhibition of $I_{Cl,swell}$ by extracellular zinc

4.1.1 $I_{Cl,swell}$ is inhibited by extracellular zinc in a concentration dependent manner

This study, specifically the use of zinc, was birthed out of the hypothesis that sulfhydryl groups play a critical role in regulating the activity of $I_{Cl,swell}$. Unpublished work by Park (2016) shows that ebselen, a molecule with high affinity for endogenous thiols, is capable of modulating $I_{Cl,swell}$. Separately, two independent studies indicate that ebselen is capable of ejecting zinc from a metallothionein binding site (Jacob et al. 1998;

Rydzik et al. 2014). In addition, work from Park (2016) has shown charged methanethiosulfonate (MTS) reagents, specifically MTSES (negatively charged) and MTSEA-Biotin (positively charged), are capable of inhibiting $I_{Cl,swell}$ when administered in the extracellular bath solution. The explicit activity of charged MTS reagents like MTSES and MTSET, which modify free or reduced thiols that are localized to the extracellular face of the membrane (Holmgren et al., 1996), identifies an extracellular sulfhydryl-specific mechanism that regulates $I_{Cl,swell}$.

The evidence of sulfhydryl group involvement in conjunction with evidence of ebselen mediated ejection of zinc led us to hypothesize that zinc, known to form zinc-cysteine complexes within protein scaffolds (Pace and Weerapana, 2014), would inhibit $I_{Cl,swell}$. Divalent cations have long been known to modify the gating of ion channels (Frankenhaeuser and Hodgkin, 1957; Gilly and Armstrong, 1982; Spires and Begenisich, 1990; Davidson and Kehl, 1995). A recent review by Noh et al. (2015) highlights the ability of zinc to bind many different types of channels, including cysteine and histidine residues of the Kv11.1 channel (Human ether-á-go-go), inhibiting its activity by causing a conformational change in the channel protein. Perhaps the most relevant example of zinc-sulfhydryl interaction in ion channels is the ability of zinc to inhibit chloride currents expressed by members of the ClC family of channels, which will be discussed in this section (Duffield et al., 2005).

Our data showed inhibition of $I_{Cl,swell}$ in the presence of extracellular zinc at 50, 100, and 300 µM in DI TNC1 astrocytes. Fitting the time course of block with a single exponential decay equation for both 100 and 300 µM zinc revealed reaction rates that had a threefold difference between them. Because the reaction rate is a product of the intrinsic

on rate constant (κ) and the reactant species concentration ($[Zn^{2+}]$), these findings imply that the variation in reaction rates depends only on the change in concentration of zinc in the bath and thus the reaction is likely first order or pseudo-first order in nature.

Empirical data suggesting a first order reaction supports the concept of a single molecule that is bound to a single site and argues against multiple binding sites that would present as a mixed-order reaction such as allosteric inhibition (Hille, 2001; Zheng & Trudeau, 2015). In this case zinc could be binding to a single specific site coordinated by multiple ligands.

4.1.2 Irreversibility of zinc mediated inhibition of $I_{Cl,swell}$ upon zinc washout

Our data indicates zinc mediated inhibition of $I_{Cl,swell}$ was not reversible within the time course of a patch clamp experiment. Upon washout of zinc from the bath solution $I_{Cl,swell}$ was not recovered, regardless of the zinc concentration used. In similar fashion, Park (2016) shows ebselen, and ebselen derivatives, as well as MTSES and MTSEA-Biotin are irreversible blockers. The apparent irreversibility mediated by zinc may be explained by mechanisms that support a strong zinc-sulfhydryl interaction.

4.1.2 (a) Chelation

Zinc readily chelates with different amino acid residues, but does so with varying affinity (Trzaskowski et al., 2007). In aqueous solution, the predominant form of zinc is $[Zn(H_2O)_6]^{+2}$ (Pesterfield, 2001; Burgess, 1978). Using a computational approach Trazskowski et al. (2007) suggest that, of all amino acid moieties, zinc's binding affinity is greatest for cysteine under physiological conditions with a Gibbs free energy of -60.4 kJ/mol. The moiety for which zinc has the next highest affinity under such conditions is histidine, with a Gibbs free energy of -25.4 kJ/mol. This large difference in calculated

Gibbs free energy implies much tighter binding of zinc to cysteine than to histidine and much slower dissociation. The experimental dissociation constant for zinc-sulfide complexes, at 25°C in water is 1.1×10^{-24} M (Yi et al., 2001), reflecting high affinity, while the zinc-histidine dissociation constant is only 8.8×10^{-13} M (Chen et al., 2000). For comparison dissociation constant of water at 25°C is $K_w = 1.03\times10^{-14}$ M.

Because chelation is dependent on binding affinities, the reversible nature of chelation varies between metal ions and their respective ligands. Irreversibility is indicative of a high association constant or a low dissociation constant as described above. Furthermore, chelation may induce conformational changes in the protein structure that inhibits dissociation.

4.1.2 (b) Protein bridging

Zinc-cysteine complexes, specifically zinc-sulfhydryl interactions, have been shown to bridge two or more protein subunits causing a conformational change in a protein. As an example, chelation of zinc by separate cysteine residues on the α-subunit and β-subunit of nitric oxide synthase (NOS3) enables dimerization and allows proper binding of a heme cofactor that is sequestered at the active site (Pace & Weerapana, 2014). Our data does not establish that zinc chelation by cysteine residues caused a conformational change that resulted in irreversible inhibition of $I_{Cl,swell}$. Nevertheless, such a conformational change induced by a zinc-cysteine interaction might sequester zinc, preventing dissociation and generating a condition of irreversibility. It is striking that our laboratory and the present work show that other sulfhydryl modifying agents are capable of irreversibly inhibiting $I_{Cl,swell}$ as well.

Supporting this hypothesis of a cation mediated block of an anion channel are findings by Duffield et al. (2005). They found that 1 mM zinc irreversibly inhibited ClC-1

muscle chloride channels in a state dependent manner with average time to block at 23 min. The group determined that zinc was capable of binding to a closed state of ClC-1 inhibiting conformational change to the open state. Furthermore, the group found that mutation of key cysteine residues of the ClC-1 channel, C277S and C278, significantly attenuated zinc block.

4.1.3 Identifying the location of zinc site of action in the signaling cascade

In an effort to elucidate where zinc binds in the cascade of $I_{Cl,swell}$ activation, we tested whether H_2O_2 applied following block of the current by zinc and zinc washout would reactivate the current. H_2O_2 is membrane permeant and is rapidly formed by superoxide dismutase from superoxide generated by NADPH oxidase and mitochondria (Brahmajothi and Campbell, 1999). Work in the laboratory has shown that activation of $I_{Cl,swell}$ by mechanical stretch, osmotic swelling, and signaling molecules including angiotensin II, endothelin, and epidermal growth factor receptor is due to ROS (Deng et al., 2016; Park, 2016; Ren & Baumgarten, 2005). Although its precise target is unknown, H_2O_2 is the most distal activator identified, and exogenous H_2O_2 activated $I_{Cl,swell}$ following block of upstream steps in the signaling cascade in cardiac myocytes as well as in DI TNC1 cells (Browe & Baumgarten, 2003; Park, 2016). Therefore, failure of exogenous H_2O_2 to reactivate $I_{Cl,swell}$ after block by zinc implies that the site of action of zinc is downstream to the target of H_2O_2 or part of the channel responsible for $I_{Cl,swell}$.

Because zinc in solution is charged, it is unlikely to passively permeate across membrane lipids. However, zinc transporters exist and 15 different "Zip" transporters that facilitate intracellular uptake of zinc have been identified (Cousins et al., 2006). These transporters may facilitate access to an intracellular site of action, and the possibility that

exogenously applied zinc blocks current by binding to an intracellular target cannot be rigorously excluded.

4.2 Voltage dependence of zinc-mediated inhibition of $I_{Cl,swell}$

The strongest evidence that zinc acts on the channel responsible for $I_{Cl,swell}$ rather than an upstream or intracellular target was that block by zinc was voltage dependent. Such voltage dependence suggest direct interaction with the channel responsible for $I_{Cl,swell}$, and most likely, a binding site within the pore the senses the electric field across the membrane (Woodhull, 1973; Hille, 2001). As outlined in the results section, the rate constant for block was 4.9-fold greater at a holding potential of -60 mV, 0.157 ± .005 $\mu M.min^{-1}$, than at a holding potential of 0 mV, 0.032 ± .001 $\mu M.min^{-1}$. This voltage-dependence can be explained by assuming the binding site for zinc senses 31% of the electric field across the pore ($\delta = 0.31$). The classical explanation for voltage-dependent block is that altering membrane potential changes the height of the free energy barrier and thereby the rate constant for block and unblock, as described for block of sodium channels (Woodhull, 1973). This analysis used an Eyring rate theory model to quantify the effect of membrane potential on channel block. The strength of the effect depends on the electrical distance, δ, which represents the fraction of the transmembrane voltage sensed at the binding site. The free energy difference due to the fraction of the electric field sensed directly alters the rate constants. Because the rate of block is the product of the rate constant and concentration of blocker at the binding site, the same result is obtained by assuming the electric field alters the concentration of the blocker and the height of the barrier and rate constants are unchanged. In the present situation, this suggests that divalent zinc was drawn into the channel pore at an inside-negative holding potential (-60 mV) and

that its concentration was elevated, increasing the rate of block. In contrast, at a holding potential of 0 mV, the absence of a transmembrane voltage gradient removed the driving force for zinc to enter the pore from the bath, reduced the zinc concentration, and thereby slowed the rate of block.

It is important to note that this discussion has focused on the effect of voltage on the rate of block by zinc and assumed that the voltage at the holding potential was responsible for altering the concentration of zinc at the binding site or, alternatively, the height of the barrier. Currents were measured, however, during a series of 550-ms test potentials. Thus, differences in holding potential altered the response at identical test potentials. Moreover, the fraction of current blocked by zinc was unaffected when initial, early and late time points were compared. These data suggest that block by zinc must have been a very slow process as compared to the duration of the voltage pulses applied. The slow onset of zinc block during repeated pulses over many minutes (e.g., Fig. 4) supports this idea.

Voltage-dependent block of channels by cations at a binding site sensing part of the electric field is a well-known mechanism of block. Cadmium (Cd^{2+}) has been shown to inhibit KCNQ1 channels in a voltage dependent manner, with an effective electrical distance across the voltage drop, δ, of approximately 40% (Tai & Goldstein, 1998). In another example, Li and Baumgarten (2000) have shown gadolinium (Gd^{3+}) block of sodium currents is also affected by voltage shifts. Both examples represent cation block of a channel at a site within the electric field.

To provide further evidence of a pore binding site for zinc, one might consider altering the chloride gradient, which would change chloride flux through the pore. A

change in the rate of zinc mediated block as a result of changing the magnitude or direction chloride gradient. In other channels, interactions between the permeant species and blocking ions have been identified and described as knock-out or knock-in of the blocker by permeant ion flux. Such studies were not conducted during the present studies. However, demonstrating knock-out or knock-in would strengthen the case for zinc block within the pore of the channel.

4.2.2 Effects of state dependence

Another possible explanation for an apparent voltage dependence of zinc block is the concept of state-dependent block. Initially characterized in detail in sodium channels (Hill et al., 1989; Wang and Strichartz, 2012; Desaphy et al., 2010), state-dependent block represents a situation where a conformational change that gates the ion channel occurs as a result of a change in membrane potential. Hill et al. (1989) show that binding of antiarrhythmics quinidine and lidocaine maintain the activated state of voltage dependent cardiac sodium channels. This conformational change results in both a change in conductance (e.g, transition from closed, to open to inactivated state) and results in an increased (or decreased) binding affinity of a blocker for its binding site or access of blocker to its binding site. Another example is block of nicotinic ACh receptors (nACh) by derivatives of local anesthetics that bind the pore of a nACh receptor in the open state, cause depolarization, and prevent the channel from closing (Steinbach, 1968; Adams, 1977; Neher et al., 1978).

This explanation might provide a plausible mechanism for the voltage dependence of zinc block of $I_{Cl,swell}$ by binding to the channel. For example, depolarization might cause a conformational change that decreases zinc affinity for the binding site as indicated

by the decreased reaction rate at more positive holding potentials. Conversely, hyperpolarization might result in a conformational change that increases zinc affinity for the binding site. However, state-dependent block is usually associated with conformational changes that alter the gating and conductance of voltage-dependent channels. Although $I_{Cl,swell}$ outwardly rectifies, it does not appear to undergo classical gating with changes in holding potential or test potentials, and time dependent tail currents on stepping back to the holding potential are absent. Rather, $I_{Cl,swell}$ behaves analogously to inward-rectifier potassium channels (K_{ir}), which display voltage-dependent currents in the absence of voltage-dependent gating. Rectification in K_{ir} channels results from voltage-dependent block by intracellular cations rather than gating of the pore.

4.3 Inhibition of $I_{Cl,swell}$ by Extracellular N-Ethylmaleimide (NEM)

Based on the chemistry of interactions between zinc and proteins, we argued that zinc is likely to irreversibly bind to a cysteine. The critical role of sulfhydryl groups in $I_{Cl,swell}$ function was confirmed by demonstrating that N-Ethylmaleimide (NEM), a sulfhydryl alkylating agent capable of generating irreversible thioether linkages, inhibited $I_{Cl,swell}$. The importance of sulfhydryl groups in channel function is not unique to $I_{Cl,swell}$. Studies on KCNQ channels show that addition of NEM irreversibly augments KCNQ2 in sympathetic neurons (Li et al., 2004). Furthermore, Duffield et al. (2005) demonstrated the importance of cysteine groups to the zinc sensitivity of ClC channels in cysteine mutagenesis studies.

Our finding that 400 µM NEM fully and irreversibly inhibited $I_{Cl,swell}$ in DI TNC1 astrocytes downstream of H_2O_2 is consistent with other recent studies in the laboratory on

the role of cysteines in $I_{Cl,swell}$. Work by Park (2016) has shown MTS reagents and other sulfhydryl modifiers also irreversibly blocked $I_{Cl,swell}$ at a site downstream of H_2O_2. H_2O_2 failed to recover $I_{Cl,swell}$ after washout of NEM, indicating an NEM site of action downstream of H_2O_2 in similar fashion to zinc and to the sulfhydryl modifying agents studied by Park (2016). Because the voltage-dependent block of $I_{Cl,swell}$ by zinc is likely to involve protein cysteines, it is appealing to raise the possibility that zinc, NEM, and other sulfhydryl reagents all act at the same site within the pore of the $I_{Cl,swell}$ channel. The data at hand strongly support the idea that each of the sites of block are downstream to the target for H_2O_2 because H_2O_2 fails to activate current after block. Nevertheless, with the conflicting evidence of NEM membrane permeability (*c.f.* Hsu et al., 2005; Von Stedingk et al. 1997) we cannot exclude the possibility that these tools modulate cysteine residues at distinct locations including some that are not a component of the protein that comprises $I_{Cl,swell}$.

4.4 Evaluation of a Common Mechanism of Divalent Mediated Inhibition of $I_{Cl,swell}$

4.4.1 Efficacy of Nickel

The inability of nickel to inhibit $I_{Cl,swell}$ at 100, 300, and 1 mM is particularly interesting. These findings may shed light on potential characteristics of the binding site and its coordination geometry. Concomitantly, the hydration shells of nickel and zinc, respectively, may play a role in explaining the differences in efficacy.

While cysteines show high affinity for zinc, and zinc finger proteins can contain cysteines exclusively, it should be noted that characteristic zinc finger motifs include histidines as well (Pace & Weerapana, 2014). Nickel has affinity for both histidine and

cysteine residues and is known to form complexes with specific geometries (Desrochers et al., 1999). Therefore, the lack of efficacy of nickel may reflect the absence of a required specific coordination geometry rather than a lack of affinity for particular residues.

The Cambridge Structural Database has identified the coordination numbers of 98 types of metal ions, including zinc and nickel, that bind to proteins. When comparing coordination to 4 or 6 ligands, nickel is found to binds to sites with a coordination number (CN) of 6 in 61% of the time (Dudev et al., 2014). In contrast zinc is bound to sites with a coordination number of 4 69% of the time. Furthermore, the prevalence of hexa-coordinated zinc is exceedingly rare and is thought to be less common than penta-coordinated zinc (Dudev et al., 2014). Taken together, our data showing that zinc irreversibly blocks $I_{Cl,swell}$ but nickel is ineffective and the reported zinc coordination geometries are consistent with the idea that zinc binds to the channel at a site with tetrahedral coordination geometry and that this predicted coordination geometry should be present in proteins postulated to represent the molecular basis of $I_{Cl,swell}$.

The hydration shell of an ion in water also plays a critical role in the interaction of the ion with a channel and in whether an ion is permeable, impermeant or a blocker (Hille, 2001). All ions are surrounded by waters of hydration in aqueous solution, adding to the perceived radius of the ion. Often, these waters must be removed, or "stripped", from the outer and all or part of the inner shell for an ion to interact with the channel's selectivity filter or other critical sites. It is striking that the rate of water substitution from the inner hydration shell of nickel, 10^4 s^{-1}, is 10,000-fold slower than that for zinc, 10^8 s^{-1} (Hille, 2001). This markedly decreased rate of water substitution around nickel may indicate that the inability to effectively strip waters of hydration limits the access of nickel to the binding

site and may contribute to the inability of nickel to block $I_{Cl,swell}$.

It is interesting to note that copper, adjacent to zinc on the periodic table, has tetrahedral coordination geometry in biological systems with affinity for thiols (Rubino & Franz, 2012). Copper has an important role as a cofactor for enzymes and is trafficked by many proteins. Assessing the efficacy of copper as a modulator of $I_{Cl,swell}$ may provide further evidence of a sulfhydryl interaction. Divalent cations with clear affinity for thiols, as well as divalents without affinity for thiols, should be tested to further evaluate the properties of the binding site.

4.4.2 Efficacy of Cadmium

Studies by Ren and Baumgarten (2005) showed that 200 µM cadmium in the bath solution blocks a rapidly inactivating component of $I_{Cl,swell}$ at positive potentials in cardiomyocytes. In our studies, addition of 200 µM cadmium to the bath solution eliminated the rapidly inactivating component at positive potentials in DI TNC1 astrocytes. The exact mechanism by which cadmium mediates this effect is unknown. Nevertheless, the presence or absence of cadmium in the bath solution did not alter the observation that zinc irreversibly blocked $I_{Cl,swell}$. Both zinc and cadmium are members of group 12 of the periodic table of elements, and thus chalcophiles, with high affinity for sulfides. That zinc does not mediate elimination of this rapidly inactivating component suggests zinc and cadmium have different mechanisms of action.

4.5 Implications

The identity of the channel protein(s) responsible for $I_{Cl,swell}$ remains unclear. SWELL1, originally believed to be a necessary component of VRAC (Qiu et al., 2014;

Voss et al., 2014), has been proven unnecessary in activating $I_{Cl,swell}$ in certain cell types (Sirianant et al., 2016). In addition, characterization of SWELL1 (Qui et al., 2014) demonstrated a total insensitivity to MTS reagents that specifically modify sulfhydryls. In contrast, zinc, which is known to be chelated by cysteine, and the sulfhydryl modifier NEM irreversibly inhibited $I_{Cl,swell}$ in native DI TNC1 astrocytes. This raises additional uncertainty regarding the proposed role of SWELL1. If zinc, which acted as a voltage-dependent inhibitor, and NEM target channel regulators rather than the channel itself, these regulators must not have been present in the system used to characterize SWELL1. On the other hand, the present findings with zinc and NEM provide additional information that must be accounted for in identifying the molecular basis for $I_{Cl,swell}$ and its regulation. These findings, in concurrence with data from our lab using MTS reagents and ebselen derivatives (Park, 2016), suggest a critical role for a site that is sensitive to exogenous sulfhydryl modifying agents. It would be ideal to take advantage of compounds that bind zinc and can be used for protein purification techniques to determine exactly where binding occurs.

4.6 Future directions

Ultimately, with a wealth of data suggesting sulfhydryl groups are responsible for modulating $I_{Cl,swell}$, the efficacy of N-ethylmaleimide is encouraging. If the affinity of a derivative maleimide compound for the binding site is great enough, one may consider the use of Alexa Fluor maleimide dyes as a thiol reactive probe. Using a combination of patch clamping techniques and fluorescence microscopy one can activate $I_{Cl,swell}$ using hypotonic challenge, or ROS inducing agents, and subsequently add a fluorescent dye-

tagged maleimide and correlate fluorescence density to current density. Furthermore, a fluorescence tag would provide imaging evidence of co-localization of the protein responsible for $I_{Cl,swell}$. Such information may indicate whether or not $I_{Cl,swell}$ is trafficked to the surface from some intracellular location, or is already membrane bound and is simply "activated" by a signaling cascade. Determining if such a localization exists would be useful for protein assays.

In the present study we observed that the divalent cation zinc is able to inhibit $I_{Cl,swell}$ in a voltage-dependent manner. This suggests an interaction with the channel protein responsible for $I_{Cl,swell}$, with an indication that zinc binds in the pore. The next step is to identify exactly where zinc binds and confirm the mechanism by which it binds.

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VITA

In Monterey, California where Noah was born, not raised-

On the basketball court is where he spent most of his undergraduate days.

He was chilling out, maxing and relaxing, while moderately schooling,

when he decided he needed to go to MCV to get moving.

Noah took a drive down to Richmond to find that patch clamping was "in"

and asked Dr. Baumgarten when he could begin.

Alas it is now that he seeks post-graduate admission,

as he aspires to accomplish many great things as a physician.