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Honors Thesis

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Abstract

Cope's gray treefrog *Hyla chrysoscelis*, accumulates and distributes glycerol as a cryoprotectant in anticipation of freezing. Transmembrane glycerol and water flux in *H. chysoscelis* erythrocytes likely occurs through HC-3, an ortholog of mammalian aquaporin 3. HC-3 protein is in higher abundance and is preferentially localized to the plasma membrane in RBCs from cold-acclimated treefrogs as compared to warm-acclimated animals. It is hypothesized that neuroendocrine agonists via receptor mediated second messenger pathways integrate signals derived from fasting, dehydration, diurnal, and/or temperature changes during cold-acclimation to regulate HC-3 expression as part of the mechanism of freeze tolerance. In this study, cultured *H. chrysoscelis* erythrocytes were exposed to 1 uM epinephrine for 30 and 60 minutes. Native HC-3 expression increased 3 fold at 30 minutes and 5.5-fold at 60 minutes relative to controls, whereas glycosylated HC-3 expression increased by 1.1-fold at 30 minutes and by 2-fold at 60 minutes relative when exposed to epinephrine. Moreover, epinephrine treatment resulted in membrane localization as compared to cytosolic distribution in control cells. Erythrocytes pre-treated with Calphostin C, a PKC inhibitor, showed no HC-3 membrane localization, and native HC-3 expression was reduced by 5% relative to controls and 3-fold relative to epinephrine-treated cells. Thus, epinephrine begins a PKC-dependent mechanism that results in an increase in HC-3 abundance, HC-3 membrane localization, and enhanced glycosylation in erythrocytes. These regulatory mechanisms are consistent with the *in vivo* regulation of HC-3 expression observed in erythrocytes from cold-acclimated treefrogs.

Dedication and Acknowledgements

I would like to dedicate this thesis to my family and friends for their support and keeping me sane throughout the process. I would like to thank Dr. Carissa Krane for accepting me as her advisee, Dr. Phil Nickell for his help along the way, and other members of Dr. Krane's lab and Dr. David Goldstein's lab at WSU. I would also like to thank the American Physiological Society and the University of Dayton Honor's Program for funding.

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Chapter 1: Literature Review

1.1. Water, Osmolarity, Regulation and Water Movement

Water is a constituent of all living things and is the major component of many organisms, contributing to approximately 60%-90% of an organism's weight (Mitchell et al., 1945). At the cellular level, this volume of water is distributed between three fluid compartments known as intracellular fluid, interstitial fluid, and blood plasma (Silverthorn et al., 2013). Water, molecular formula H_2O, is a tetrahedron-shaped molecule with unequal electron distribution between oxygen and two hydrogen atoms. This unequal sharing results from oxygen's electronegativity. The uneven charge distribution allows water to retain polar asymmetry with both positively and negatively charged regions. Because of the unique biological chemistry of the molecule, water possesses significant properties without which the existence of life would not be possible. These pertain to water's electrostatic, cohesive, and solvent characteristics (Bensen, 2008).

Proper fluid regulation is essential for an organism's homeostasis and life processes. External variables such as diet, health, hydration, temperature, and other factors contribute to oscillations of body fluids affecting homeostasis (Anderson, 1997; Bourque, 2008; Krane and Goldstein, 2007). Organism health is at risk by deviation of extracellular fluid from homeostatic levels which affects tissue and cell function, and biological activity of essential macromolecules (Steenbergen and Jennings, 1985; Strange, 2004). Therefore, efficient and reliable mechanisms to monitor detrimental

fluctuations in fluid volume and composition are necessary for organisms to sustain healthy body functions. Over the course of evolution, multicellular organisms have developed a multitude of sensory and response strategies for changes in fluid volume and composition. For example, certain mammals have evolved central osmoreceptors found in cerebral tissue and peripheral osmoreceptors within alimentary tract and blood vessels, and osmolarity-sensitive prolactin cells in tilapia, *Oreochromnis mossambicus,* have evolved (Bourque, 2008; Weber et al., 2004). These examples represent two instances where animals have evolved varying sensory techniques to aid in osmoregulation.

Water's ubiquitous distribution among both complex and single cell organisms coupled with its unique features, allows this molecule to provide crucial roles in biological systems. For instance, the role of water as a universal solvent is a result of its polar nature. Clusters of water molecules that form around individual ions or molecules are known as hydration shells. These hydration shells allow soluble substances to dissolve and remain in the fluid phase, altering the concentration of a solution (Bensen, 2008; Silverthorn et al., 2013). In biological solutions this concentration is known as osmolarity, the number of osmotically active particles of ions/molecules per liter of solution. Consequently, water moves across the barrier separating solutions in response to the concentration gradient of the active particles (Silverthorn et al., 2013).

Osmoregulation is a vital, cohesive process that regulates osmotic pressure via control of water and ionic composition of extracellular fluids surrounding a cell (Bourque, 2008). In a compartmentalized system, the movement of water across a

selectively permeable membrane, which allows passage of solvent only is known as osmosis (Bensen, 2008; Silverthorn et al., 2013; Strange, 2004). This phenomenon occurs as a result of a solute concentration gradient in which water moves to dilute a higher concentration solution from a lower one. Therefore, solutions separated by a semipermeable membrane will reach equilibrium with respect to their molecular concentrations via movement of solvent in certain laboratory conditions. However, in many biological systems *in vivo,* osmosis drives water movement across at membrane barrier where osmotic equilibrium is attained, but chemical equilibrium is not. Osmosis utilizes the movement of water, thus a force can be applied to prevent and oppose solvent movement into a highly concentrated solution known as osmotic pressure (Bensen, 2008). Osmoregulation plays a critical role in fluid homeostasis by maintaining osmotic equilibrium between the intracellular and extracellular fluid.

Within an organism, water follows the same mechanics of osmosis which was previously stated. The phospholipid bilayer of the cell membrane serves as a selectively permeable membrane. The lipid bilayer allows water to move freely between intracellular and extracellular fluid if a solute concentration gradient is present. Osmotically driven water flux can occur via simple diffusion as it travels through the lipophilic lipid bilayer. However, water is a polar molecule and simple diffusion through a nonpolar membrane; therefore, simple diffusion is not an adequate mode of transportation for physiological events that require fast, bulk movements of water. Consequently, in events such as these, transmembrane water movement is facilitated through protein water channels known as aquaporins (Krane and Goldstein, 2007).

1.2. Aquaporins

The cell membrane functions as a barricade between the cell and the surrounding extracellular fluid. As previously stated, water is free to cross this semipermeable membrane by osmosis in order to maintain osmotic equilibrium. However, the cell membrane is simply not a static barrier. Accordingly, cell membrane fatty acid, cholesterol, phospholipid, and membrane protein composition can be modified in numerous amounts of living cells (Spector and Yorek, 1985; Verkman, 2000). Furthermore, these membrane modifications are robust enough to alter membrane fluidity and permeability. The extent of these alterations vary from cell type to cell type, therefore the water permeability of cell membranes within the body differs greatly based on cellular function (Spector and Yorek, 1985). For instance, the water permeability of mammalian erythrocytes is significantly greater than that of other cell types (Finkelstein, 1987). In mammals, the permeability of oral epithelia is markedly different than the permeability of epidermis cell counterpart. It is believed the determinant factor for these permeability differences are due to membrane composition (Squier, et al., 1991).

The presence of water-channel proteins found within the membrane is one membrane alteration that allows membrane permeability of water (Lodish et al., 2000). These protein aquaporins exist in cell membranes to increase water permeability and water movement across membranes (Borgnia et al., 1999). In 1991, Dr. Peter Agre and colleagues successfully identified and cloned an aquaporin, AQP1, from human erythrocytes originally known as CHIP28 (channel-like integral protein of 28-kDa (Preston and Agre, 1991). CHIP28 was shown to increase membrane permeability in

response to osmotic gradients. Initial studies performed by Agre and colleagues revealed that the AQP1 protein is comprised of 269 amino acid residues (Preston and Agre., 1991). Aquaporins are integral protein water channels that belong to the MIP (major intrinsic protein) superfamily, near 28-kDa in size, and assemble as homotetramer where each monomer is a functional water pore. Aquaporins also resemble the MIP superfamily by the characteristic six transmembrane regions, with both amino and carboxyl terminal ends located within the cytoplasm. Additionally, this six-transmembrane protein structure allows the aquaporin to form a pore which allows for water movement through the cell membrane (Mulders et al., 1995; Krane and Goldstein, 2007). According to Jung et al. (1994), the aquaporin structure possesses three unique membrane spanning domains and retains two loops referred to loop B and loop E. Loop B is located on the cytoplasmic side whereas loop E is found on the extracellular side. Additionally, both loops contain identical amino acid sequence of asparagine-proline-alanine (NPA). Although found on opposing sides of the cell membrane, these loops connect through the membrane to form a pathway narrow enough for individual water molecules to pass through (Jung et al., 1994). The narrow pore region is created by interactions of amino acids found within the pore (His 182, Arg 197, Cys 191, Phe 58), resulting in a 2.8-A diameter opening (Sui et al., 2001). It is observed that intermolecular hydrogen bonds between adjacent water molecules must be broken so individual water molecules can pass through continuously. It is important to note that aquaporins do not transport water across the cell membrane. Water movement is driven by the osmotic gradient between intracellular and extracellular fluids; aquaporins provide the channel by which water molecules will move through.

1.3. Aquaglyceroporins

As previously described, some aquaporins, such as AQP1, are permeable protein channels that strictly facilitate water movement. However, some members of the aquaporin family do not follow this same blueprint. For instance AQP3, similar to AQP1, is permeated by water, glycerol and small solutes (NH_3 and NH_4^+) and allow these molecules to move in and out of the cell (Borgnia et al., 1999; Krane and Goldstein, 2007). Because of its permeability for water as well as glycerol, AQP3 is referred to as an aquaglyceroporin. Aquaporins with similar functions to AQP3 comprise a second functional class of MIP proteins known as aquaglyceroporins (GLPs).

By utilizing a homology cloning approach, three scientific groups successfully isolated complementary DNA (cDNA) encoding the AQP3 protein product (Ishikawa et al., 1994; Echevarria et al., 1994; Ecelbarger et al., 1994). Although AQPs and GLPs have similar permeability functions, GLPs differ from AQPs structurally. One of the most extensively studied members of the GLP, GlpF, assembles into an aquaporin structure loosely similar to AQP1. Structural differences in GlpF reveal the channel retains asymmetric form, a differing extracellular loop length, and altered amino acids within the constriction region of the narrow pore region (Borgnia et al., 1999, Krane and Goldstein, 2007). More specifically, Froger et al. (1998) revealed five amino acid changes in the sequence of GlpF that are different from AQP1: one in the third transmembrane helix, two in the loop E, and two in the sixth transmembrane helix (Froger et al., 1998). These structural differences allow GLPs to resemble aquaporins and facilitate transmembrane water and solute movement via concentration gradients.

1.4. Aquaporins and Aquaglyceroporins in Mammals

Protein members which reside in the aquaporin family are thought to be protein-forming water channels with similar overall structural design like AQP1. However, as previously mentioned, the classes of the MIP family have structural differences for functional purposes at the cellular level. As of now, there are thirteen known aquaporin family members found within mammals: AQP0-AQP12. These aquaporins are responsible for water, organic compound, and small solute movement between the cell and surrounding environment (Borgnia et al., 1999; Krane and Goldstein, 2007). Through evolutionary comparison of sequences within MIP family members, AQP0, AQP1, AQP2, AQP4, AQP5, AQP6, and AQP8 serve as proteins in the water-selective subgroup, while AQP3, AQP7, AQP9, and AQP10 are grouped in the aquaglyceroporin subclass (Krane and Goldstein, 2007).

Aquaglyceroporins facilitate the movement of water, glycerol, urea and other small solutes (King et al., 2004). Therefore, these GLPs are found in various locations throughout the mammalian body and localized in the membranes of cells which function in transmembrane glycerol flux. The production of glycerol results from hydrolysis of triacylglycerol which occurs in adipocytes. Upon production, glycerol is released from adipocytes into blood plasma and is subsequently taken up into the liver. It has been reported that AQP7 is found in membranes of adipocytes which allows for glycerol efflux (Kuriyama et al., 2002), and hepatocytes employ the use of AQP9 to facilitate glycerol influx (Kuriyama, et al., 2002). Additionally, AQP7 and AQP9 are localized in other regions throughout the body such as testis and skeletal muscles, and brain and lung,

respectively. In addition, AQP3 is also extensively expressed throughout the body in the gastrointestinal tract, erythrocytes, epidermis and the brain (Borgnia et al., 1999; Krane and Goldstein, 2007; Yasui et al., 2008).

1.5. Aquaporin and Aquaglyceroporin: Expression and Membrane Localization

Controlling the abundance of proteins within a cell is an essential process that occurs through a wide range of mechanisms. Cells increase or decrease target protein products through regulation of gene expression. Because members of the aquaporin family are proteins and contribute to cell membrane permeability, their regulation is crucial for homeostasis in both plants and mammals (Kjellbom et al., 1999). Therefore, aquaporin gene expression is a tightly regulated process that can have many physiological triggers. For instance, the genes encoding aquaporin products are subject to temporal and tissue-selective regulatory expression mechanisms via control of transcription and translation, post-translational modifications and membrane translocation (Krane and Goldstein, 2007).

Aquaporin family gene expression has been regulated through signals such as cyclic adenosine monophosphate (cAMP), cyclic guanosine monophosphate (cGMP), nitric oxide (NO), hypertonicity as well as others. The water permeability of AQP3 and AQP6 are regulated by the extracellular fluid and are pH-dependent water channels (Krane and Goldstein, 2007). In a response to dehydration, AQP2 mRNA and protein abundance have been shown to be upregulated in the principal cells of the collecting duct (Borgnia et al., 1999). In addition, apical membrane insertion of AQP2 is regulated

temporally by vasopressin-induced G-protein coupled cell signaling (Borgnia et al., 1999). Unlike AQP2, AQP1 lacks this short term regulation as it is constantly expressed in membranes of high water permeability. It has also been speculated that aquaporin activity is also shown to be regulated through "gating" via the phosphorylation of conserved serine and threonine amino acid residues shared by all aquaporin family members, and the presence of divalent cations (Törnroth-Horsefield et al., 2010). Although the mechanisms of aquaporin regulation are widespread and numerous, these aforementioned examples are used to illustrate the diversity in AQP/GLP regulation.

In order for AQPs and GLPs to contribute to the passage of water and solutes through the cell membrane, these transmembrane proteins must be inserted into the membrane. In order for this vital step to occur, aquaporins and aquaglyceroporins must be trafficked to the cell membrane in order for their subsequent insertion to take place. Previous studies have focused on cell signaling for mammalian aquaporin membrane translocation, however aquaglyceroporin trafficking are not as clearly understood. Regulatory studies on AQP2 in collecting duct principal cells reveal a cell signaling pathway initiated by antidiuretic hormone, vasopressin (Borgnia et al., 1999; Brown et al., 1998; Krane and Goldstein, 2007). In principal cells of the kidney, AQP2's presence in the membrane is tightly regulated as it is originally found within intracellular vesicles. In 1981, Wade et al., demonstrated these AQP2-containing vesicles are trafficked to the cell membrane by vasopressin action. Further investigation into this pathway revealed AQP2 translocation from cytosol to the membrane is a protein kinase A (PKA) dependent mechanism via G protein activation (Wade et al., 1981). Upon vasopressin binding a G-

protein coupled receptor, adenylate cyclase is activated, producing the second messenger cAMP. Production of cAMP leads to the downstream activation of PKA which phosphorylates AQP2, regulating vesicular movement and insertion into the membrane (Brown et al., 1998).

In rats, it has been observed that epithelial cells of the bile duct which possess secretin receptors, exhibit AQP1 translocation from the cytosol to the membrane following secretin secretion (Marinelli et al., 1997). Therefore, it was thought that AQP1 membrane localization followed a secretin-dependent pathway. It was also believed that phosphorylation of AQP1 could increase cell membrane permeability. However, it was not understood whether this protein kinase phosphorylation resulted in AQP1 trafficking or increased function of the aquaporin. Conner et al. (2010) exposed cells to hypotonic conditions and observed a significant decrease in AQP1 membrane localization upon administration of protein kinase C (PKC) and microtubule inhibitors (Conner et al., 2010). These same results were not observed upon exposure of PKA and actin inhibitors, thus, AQP1 membrane localization occurs through PKC activity along microtubules of the cytoskeleton.

In addition, different vesicular trafficking mechanisms have also been discovered for aquaglyceroporins. Aquaglyceroporins, the second functional class of MIP, utilize different pathways for membrane translocation. Mammalian AQP3 is a well-studied aquaglyceroporin protein. AQP3 gene transcription is regulated by the hormone epinephrine. AQP3 mRNA expression has been shown to increase in response to epinephrine in Caco-2 cells (Asai et al., 2006). The AQP3 gene regulation via

epinephrine may provide evidence for the mechanism of AQP3 translocation through epinephrine receptors through G-protein coupled receptors. In Caco-2 cells, phospholipase C and PKC inhibition were shown to decrease AQP3 trafficking induced by epinephrine, while adenylate cyclase and PKA inhibitors had no effect on trafficking (Yasui et al., 2008). Results from these experiments suggest a possible signal transduction mechanism, G protein coupled receptor-PLC-PKC, which is responsible for membrane translocation of AQP3 induced by epinephrine.

1.6. Freeze Tolerance

Environmental factors are crucial for animals to survive and affect the regulatory maintenance of homeostasis. Animals found within the natural environment are exposed to various changes in the environment such as lack of food, lack of water, and temperature extremes. Many of these changes occur as a result of seasonal changes. In order to maintain regular body functions in varying environmental conditions, animals need to respond physiologically to maintain homeostasis. For instance, many temperate zone ectototherms are challenged with cold temperatures which act as a stress stimuli for the organism (Costanzo et al., 1992). One physiological mechanism utilized by these ectotherms to survive freezing temperatures is known as freeze avoidance in which organisms use supercooling techniques to prevent the freezing of bodily fluids (Layne and Lee, 1987).

Freeze tolerance, another physiological survival mechanism is used in a variety of organisms, including snakes, insects, fish, and amphibians to survive in harsh, freezing

conditions (Wells, 2007; Zachariassen, 1985). Freeze tolerance is an overwintering adaptation that allows select organisms to survive sub-freezing temperatures as a result of seasonal change by coping with natural episodes of freezing of body fluids (Costanzo, et al., 1992). Similar freeze tolerant strategies have also been identified in amphibians found in temperate locations that must cope with changing temperatures (Costanzo et al., 1992).

Freeze tolerance and cryprotectant mechanisms have evolved in animals in order to address the problem of freezing of internal body fluids. If not regulated, freezing of internal body fluids can be detrimental to an animal's survival. The freezing of living cells is a two-fold problem (Mazure, 1988). Cells must maintain fluid homeostasis while cooling to sub-freezing temperatures as well as during the warming process in order to survive (Mazure, 1988). Without protective measures, these cooling and warming processes may disrupt the cell and cause death via freezing of extracellular fluid. For instance, when a cell is subject to freezing temperatures, ice crystals begin to form in the extracellular fluid surrounding the cell. Only water molecules contribute to the ice crystal formation, and ion such as Na^+, Ca^{2+}, and other non-penetrating solutes remain in the unfrozen solution. This results in an increasingly concentrated extracellular fluid, altering osmotic equilibrium. Water moves by osmosis from inside the cell to outside of the cell into the hypertonic extracellular fluid. This exchange of water can induce lethal complications upon the cell due to shrinkage, membrane disruption, and failure to maintain homeostasis (Layne and Lee, 1995).

Therefore, freeze tolerant organisms attempt to control/eliminate these unwarranted effects of freezing through the use of cryoprotectants. The types of

cryoprotectants found within different organisms are extensive and consists of methanol, glycerol, dimethyl sulfoxide, low molecular weight sugars, and polyhydric alcohols, as well as others (Layne and Lee, 1995; Mazure, 1988). Because rapid cooling is shown to invoke injuries to cells, cryoprotectants such as glycerol allow a cell to achieve a state of gradual cooling (Costanzo el al., 1992). Cryoprotectants also reduce the severity of cellular dehydration based on water movement into extracellular fluid, and reduce the overall amount of ice forming. The reduction of ice formation and water freezing point are created by cryoprotectants through association with colligative properties of a solution such as the effect of solute (cryoprotectant) concentration within an aqueous environment (Layne and Lee, 1995; Layne, 1999). In order to combat the effects of extracellular freezing, the organism distributes the cryoprotectant throughout the body to elicit the aforementioned effects upon the initial freezing of exterior body.

However, in order for cryoprotection to work effectively, freeze tolerant organisms must first synthesize or acquire cryoprotectants. It is believed that animals can accumulate cryoprotectants through a process known as cold-acclimation (Lotshow, 1977). In a response to changes in environmental factors, animals become acclimated to the cold environment through behavioral and physiological alterations. For instance, ectothermic organisms that undergo a period of cold-acclimation, tend to decrease muscle activity, metabolism, and heart rate, and slow overall metabolism. These behavioral changes act as stimuli for certain sensory cells within the organism to allow for the production or accumulation of cryoprotectant. The cryoprotectant is then distributed throughout tissues and organs and at the cellular level, water and cryoprotectant move in

and out of cells in order to preserve osmotic equilibrium during freezing and thawing.

1.7. *Hyla chrysoscelis*

As previously mentioned, fish, amphibians, snakes, and insects employ the aforementioned mechanism of freeze tolerant in order to survive in freezing conditions. Terrestrial amphibians are one type of organism that may be faced with the possibility of freezing. To face this problem, a select subset of these amphibians have evolved the ability tolerate freezing of a substantial amount of their extracellular fluids. Upon days of thawing, these organisms retain nearly 65% of their body water as ice (Layne and Lee, 1987). Studies reveal at least six species of North American frogs capable of freeze tolerance; *Hyla chrysoscelis, Hyla versicolor, Pseudacris crucifer, Pseudacris triseriata, Hyla regilla,* and *Rana sylvatica* (Costanzo et al., 1992; Croes et al., 2000; Layne and Lee, 1995). These different treefrogs use cryoprotectants in order to maintain cell integrity during the process of cooling and warming. For instance, *H. chrysoscelis* and *H. versicolor* use glycerol as cryoprotectant, whereas *R. sylvatica, P. triseriata, H. regilla,* and *P. crucifer* utilize glucose (Costanzo et al., 1992; Layne, 1999).

Hyla chrysoscelis, also known as Cope's gray treefrog, is located in central, southeastern, and northeastern United States, as well as some areas of Canada (Layne and Lee 1995). During the winter months, *H. chrysoscelis* survives by gradually freezing and reducing metabolic activity (Layne and Lee, 1995; Layne, 1999). *H. chrysoscelis* undergoes a cold-acclimation process in order to prepare for freezing of body fluids prior to the exposure of sub-freezing temperatures during winter. Environmental changes such

as a decrease in food and water abundance/availability, and a change in the light cycles occur during seasonal change. These environmental changes may play a role in freeze tolerance observed in *H. chrysoscelis* via behavioral changes in the organism as a response.

One essential physiological change observed in *H. chrysoscelis* during cold-acclimation is the reduction in glomerular filtration rate and urinary flow which is thought to occur in order retain glycerol (Zimmerman et al., 2007). The concentration of circulating glycerol within the treefrog also increases in order to accumulate cryoprotectant. Dehydration stress, as a result of the changing external environment, is believed to trigger glycogenolysis in freeze tolerant treefrogs. Glycogenolysis occurs in the liver and results in the production of glycerol that is released into circulation and distributed throughout the body (Layne, 1999). Thus, glycerol is synthesized in the liver and exits hepatocytes in order to enter various cells throughout *H. chrysoscelis*. As a result, glycerol levels are shown to be elevated at high concentrations in cold-acclimated treefrogs in preparation to freezing conditions (Zimmerman et al., 2007). The presence of glycerol also functions to regulate osmotic equilibrium to control water movement between intracellular and extracellular fluids.

In order for proper fluid homeostasis to be maintained during cooling and warming, glycerol and water must be effectively transported between the intra- and extracellular fluids. It is hypothesized that aquaporins are responsible for transmembrane water movement and aquaglyceroporins allow transmembrane flux of organic glycerol.

Many of these aquaporin water channels have been identified in amphibians (Abrami et al., 1994; Abrami et al., 1995; Pandey et al., 2010; Zimmerman et al., 2007). To date, three members of the MIP have been identified in *H. chrysoscelis* which are homologs of mammalian AQP1, AQP2, and AQP3, respectively: HC-1, HC-2, and HC-3. HC-1 and HC-2 have been identified as aquaporins, while HC-3 is a functional aquaglyceroporin (Zimmerman et al., 2007; Pandey et al., 2010; Mutyam et al., 2011). Because HC-3 is a GLP, it is thought to be involved in freeze tolerance in *H. chrysoscelis.* Previous studies show an increase of HC-3 expression in erythrocytes from cold-acclimated treefrogs when compared to warm-acclimated (Goldstein et al., 2010). This suggests HC-3 is responsible for the transmembrane movement of glycerol in cells of cold-acclimated *H. chrysoscelis*, enhancing cryoprotective effects during the freezing process.

1.8. Hypothesis

Although the mechanism for HC-3 membrane translocation remains unknown, Mutyam et al. (2011) observed HC-3 membrane localization to be more prominent in erythrocytes from cold-acclimated *H. chrysoscelis* as compared to warm-acclimated *H. chrysoscelis*. These data suggest the mechanism responsible for HC-3 translocation to the cell membrane occurs *in vivo* in cold-acclimated *H. chrysoscelis.* This thesis project utilized a *H. chrysoscelis* erythrocyte culture system designed by Muytam et al. (2011) to further investigate HC-3 protein expression and identify the mechanism responsible for HC-3 membrane localization in cold-acclimated treefrogs. We hypothesize that neuroendocrine hormones, which may be responsible for physiological changes in *H.*

chrysoscelis during cold-acclimation, act through the second messenger pathways to

regulate HC-3 expression in erythrocytes as part of the mechanism of freeze tolerance.

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Chapter 2: Epinephrine regulates aquaglyceroporin HC-3 expression and subcellular localization in cultured erythrocytes from the freeze-tolerant treefrog, *Hyla chrysoscelis*

I.   Abstract

Cope's gray treefrog, *Hyla chrysoscelis*, accumulates and distributes glycerol as a cryoprotectant in anticipation of freezing. Transmembrane glycerol and water flux in *H. chysoscelis* erythrocytes likely occurs through HC-3, an ortholog of mammalian aquaporin 3. HC-3 protein is in higher abundance and is preferentially localized to the plasma membrane in RBCs from cold-acclimated treefrogs as compared to warm-acclimated animals. It is hypothesized that neuroendocrine agonists via receptor mediated second messenger pathways integrate signals derived from fasting, dehydration, diurnal, and/or temperature changes during cold-acclimation to regulate HC-3 expression as part of the mechanism of freeze tolerance. In this study, cultured *H. chrysoscelis* erythrocytes were exposed to 1 uM epinephrine for 30 and 60 minutes. Native HC-3 expression increased 3 fold at 30 minutes and 5.5-fold at 60 minutes relative to controls, whereas glycosylated HC-3 expression increased by 1.1-fold at 30 minutes and by 2-fold at 60 minutes relative when exposed to epinephrine. Moreover, epinephrine treatment resulted in membrane localization as compared to cytosolic distribution in control cells. Erythrocytes pre-treated with Calphostin C, a PKC inhibitor, showed no additional HC-3 membrane localization, and native HC-3 expression was reduced by 66% relative to controls and 94% relative to epinephrine-treated cells. Thus, epinephrine begins a PKC-

dependent mechanism that results in an increase in HC-3 abundance, HC-3 membrane localization, and enhanced glycosylation in erythrocytes. These regulatory mechanisms are consistent with the *in vivo* regulation of HC-3 expression observed in erythrocytes from cold-acclimated treefrogs.

II.   Introduction

Animals that inhabit regions were freezing/subfreezing temperatures occur are faced with the potential issue of their bodily fluids freezing during the winter. One evolutionary adaptation in animals to address the freezing of body fluids is known as freeze avoidance. Antarctic fishes and a few terrestrial invertebrates carry out freeze avoidance through supercooling mechanisms that allow the organism to decrease the freezing point of its bodily fluids to prevent the fluids from freezing (Costanzo et al., 1992). Other organisms, such as Cope's gray treefrog *Hyla chrysoscelis,* survive subfreezing conditions through the use of freeze tolerant mechanisms to cope with the freezing of body fluids (Layne and Lee, 1995; Mazure, 1988). Unlike freeze avoidance, freeze tolerance allows *H. chrysoscelis*, and other organisms, to survive freezing of their bodily fluids (Layne and Lee, 1995). *Hyla chrysoscelis* utilizes freeze tolerance as a survival mechanism during the winter months via the accumulation and distribution of glycerol (Costanzo et al., 1992; Layne and Lee 1995; Zimmerman et al., 2007). In *H. chrysoscelisis*, glycerol acts as a cryoprotectant to stabilize protein and/or membrane structures. Cryoprotectants stabilize the plasma membrane by preventing the irreversible transition to the gel state of the membrane during cell volume reduction. In addition, cryoprotectants also provide stability by interacting with the polar head groups of membrane lipids (Mazure, 1988; Storey, 1997). In addition, cryoprotectants aid in osmotic regulation of water between intra- and extracellular fluids to maintain fluid homeostasis by raising the osmolality of body fluids (Storey and Storey,1992; Storey, 1997). Increasing the osmolality of the extracellular fluid reduces the amount of total

body water that can freeze and prevents intracellular fluids from falling below a critical

minimum volume.

In order to prepare for freezing, *H. chrysoscelis* synthesizes glycerol in

hepatocytes of the liver. After production, the glycerol is then released into circulation

where it is distributed throughout tissues in *H. chrysoscelis.* It has been observed in *Hyla*

versicolor as well as *H. chrysoscelis,* that extracellular glycerol concentrations increase in

cold-acclimated frogs as compared to warm-acclimated treefrogs (Storey and Storey,

1985; Costanzo et al., 1992). Moreover, in *H. chrysoscelis* it has been observed that

extracellular glycerol concentration may remain at high levels for an extended period of

time if cold-acclimated organisms are continuously exposed to cold temperatures (Irwin

and Lee, 2003). In order for cryoprotection to work effectively, *H. chrysoscelis* cells need

to accumulate the freely circulating glycerol in the extracellular fluid. Although glycerol

synthesis in the liver leads to an increase in freely circulating glycerol, the cryoprotectant

must then be transported across the cell membrane of cells to maintain fluid homeostasis

during freezing and thawing (Costanzo et al., 1992).

It is hypothesized that one route for this essential transmembrane movement of

glycerol occurs through protein forming water channels from the major intrinsic protein

(MIP) family known as aquaporins (Preston and Agre, 1991; Thomas et al., 2002). An

aquaporin is an integral membrane protein with a molecular weight near 28-kDa. The

protein forms a channel through the membrane insertion of six membrane spanning

domains, with intracellular amino and carboxy termini. Monomers of the aquaporin

assemble as homotetramers in the membrane though each monomer is a functional water pore (Borgnia et al., 1999; Krane and Goldstein, 2007; Mulders et al., 1995). Aquaporins (AQPs) comprise one class of MIP members and are permeated only by water molecules, while a second MIP class, aquaglyceroporins (GLPs), is permeated by water and small solutes such as glycerol (Borgnia et al., 1999; Krane and Goldstein, 2007; Thomas et al., 2002). Because of its selective permeability for glycerol, it is likely that a GLP is involved in the transmembrane movement of cryoprotectant glycerol between intra- and extracellular fluids.

A number of AQPs and GLPs have been identified in amphibians (Abrami et al., 1994; Abrami et al., 1995). Specifically, three AQPs/GLPs were isolated and identified in *H. chrysoscelis* which are homologs of mammalian AQP1, AQP2, and AQP3, respectively: HC-1, HC-2, and HC-3. HC-1 and HC-2 have been classified as aquaporins permeable to water, while HC-3 is a functional aquaglyceroporin (Zimmerman et al., 2007). Previous studies have shown increased expression of HC-3 mRNA and protein in erythrocytes from cold-acclimated treefrogs, in comparison to warm-acclimated treefrogs (Goldstein et al., 2010; Pandey et al., 2010). Moreover, Goldstein et al. (2010) observed erythrocytes placed in 250mOsm glycerol containing media experienced cell lysis, which is indicative of glycerol permeability via HC-3. Furthermore, the glycerol permeability, observed as cell lysis, was inhibited using $HgCl_2$, a known AQP/GLP channel inhibitor (Goldstein et al., 2010). Also, Mutyam et al. (2011) observed erythrocytes cultured in media containing glycerol show increased abundance of glycosylated HC-3 as well as enhanced plasma membrane translocation.

This membrane localization of HC-3 in cold-acclimated *H. chrysoscelis* suggests the aquaglyceroporin must be translocated from cytosol to the cell membrane to enhance glycerol permeability. Combined, these data suggest a role for HC-3 in freeze tolerance via facilitation of transmembrane glycerol flux.

However, the link between the environmental stimuli and HC-3 membrane translocation to the cell membrane in *H. chrysoscelis* erythrocytes remains unknown. Moreover, in mammals, previous studies have identified membrane trafficking mechanisms of numerous aquaporins. For instance, in principal cells in the kidney, AQP2 expression in the cell membrane is tightly regulated by vesicular trafficking of the aquaporin by a vasopressin induced protein kinase A (PKA) pathway (Borgnia et al.,1999; Brown, 1998;). In addition, membrane translocation of AQP1 in epithelial cells of the bile duct in rats is triggered by secretin (Conner et al., 2010; Marinelli, et al., 1997). Furthermore, Ishikawa et al. (2005) discovered that stimulation of the muscarinic acetylcholine receptor (mAChR) leads to AQP5 membrane trafficking to the apical membrane of cells in the parotid gland of rats. Also, it is reported that epinephrine is responsible for AQP3 membrane translocation via a GqPCR-PLC-PKC dependent pathway in Caco-2 cells. Lastly, epinephrine has also been shown to increase expression of AQP3 mRNA is Caco-2 cells as well (Asai et al., 2006; Yasui et al., 2008).

Although the mechanism for HC-3 membrane translocation remains unknown, Mutyam et al. (2011) observed HC-3 membrane localization to be more prominent in erythrocytes from cold-acclimated *H. chrysoscelis* as compared to warm-acclimated *H.*

chrysoscelis. These data suggest the mechanism responsible for HC-3 translocation to the cell membrane occurs *in vivo* in cold-acclimated *H. chrysoscelis.* It is known that *H. chrysoscelis* undergoes a period of cold-acclimation in preparation for freezing. Several environmental changes such as decreased abundance of food/water, changes in light cycles, and reduced temperature occur as a result of seasonal change into winter months. *H. chrysoscelis* undergoes behavioral and physiological changes such as reduced glomerular filtration rate and urinary flow, as well as glycogenolysis which likely occur as a result of environmental stimuli (Layne, 1999; Zimmerman et al., 2007).

This thesis project utilized a *H. chrysoscelis* erythrocyte culture system designed by Muytam et al. (2011) to further investigate HC-3 protein expression and identify the mechanisms responsible for HC-3 membrane localization in cold-acclimated treefrogs. We hypothesize that neuroendocrine hormones which may be responsible for physiological changes in *H. chrysoscelis* during cold-acclimation, act through second messenger pathways to regulate HC-3 expression in erythrocytes as part of the mechanism of freeze tolerance.

III.   Material and Methods

Hyla chrysoscelis

Male *Hyla chrysoscelis* were wild caught from Southwest Ohio as noted in
Zimmerman et al. (2007). Treefrogs were stored in Dr. David Goldstein's laboratory at
Wright State University. Warm-acclimated treefrogs were exposed to natural light cycles
(12:12-hr) and kept at ambient temperature (23°C) throughout the summer. The frogs
were acclimated to 5°C for over two months, and throughout the study, frogs were fed
crickets three times per week and water was available in cages *ad libitum* as described by
Mutyam el al. (2011). The collection, housing, and experimental protocols for the use of
H. chrysoscelis were approved by the Institutional Animal Care and Use Committee
(IACUC) at Wright State University.

Hyla chrysoscelis Erythrocyte Cell Culture

Mutyam et al. (2011) previously established cell culture conditions necessary for
the maintenance of *H. chrysoscelis* erythrocyte cultures. Blood was drawn from the
trunk/brachial artery of male *H. chrysoscelis* and was collected in heparinized capillary
tubes. Remaining freshly isolated erythrocytes were put in a 15 mL conical tube
containing 10 mL complete cell culture media (CCCM (250mOsM): RPMI 1640 medium
supplemented with L-glutamine (Invitrogen Carlsbad, CA), 100 units/mL amphotericin B
(Invitrogen), 5% fetal bovine serum (Fisher Scientific, Hanover Park, IL). Erythrocytes
(7×10^6) were resuspended in CCCM or CCCM containing 0.150 M glycerol
(400mOsM) in 7 mL. Cells were cultured in Corning flasks and shaken at 200 rpm at

room temperature for the duration of the experiment. Media was replenished every 24 hours and trypan blue dye was used to determine cellular viability cultures.

Signal Transduction Treatments

After 48 hours in culture, erythrocytes cultured in CCCM were exposed to 1μM epinephrine (Sigma-Aldrich, E4642) for 30, 60, and 90 minutes; separate erythrocyte cell cultures were exposed to 1μM cAMP (Simga-Aldrich, D0269) for 30, 60, and 90 minutes and shaken at 200 rpm at room temperature for the duration of agonist exposure. Select cultures were subject to pre-incubation with 1 μM calphostin C (Sigma-Aldrich, C6303), a PKC inhibitor, for 60 minutes prior to the addition of 1 μM of epinephrine.

Protein Isolation and Quantification

A freshly isolated protein sample was collected by washing 10 μL of the freshly isolated cells in cold 1xPBS, and pelleted by centrifugation at 1000 x g. The pellet was resuspended in 35 μL isolation solution and subjected to a three freeze-thaw cycles (1 minute dry ice and 1 minute 37°C dry heat bath). Cells ($1x10^6$) were removed from erythrocyte cultures for subsequent time points at overnight in culture, and 48 hours in culture. In addition, $1x10^6$ cells were also removed from erythrocyte cultures after exposure to agonist for different exposure lengths. Cells were pelleted out at 1,000 x g for approximately ten minutes. CCCM supernatant was removed and the remaining erythrocytes were resuspended in 35μL of protein isolation solution (5 mM triethanoalamine, 125 mM sucrose, 1:1,000 protease inhibitor cocktail (PIC) (Sigma-Aldrich), and 2 mg/mL phenylmethylsulfonyl fluoride (PMSF) (Sigma-Aldrich). Three

freeze-thaw cycles were used to lyse the cells as previously mentioned. Pierce

bicinchoninic (BCA) protein assay kit (ThermoScientific, Waltham, MA) was used to

determine the protein concentrated of the isolated protein sample.

Western Blot Analysis of HC-3 Expression

        Western blotting was performed as previously described (Krane et al., 1999).

Approximately $1x10^6$ erythrocytes were collected from cell cultures at select time points

during agonist and inhibitor treatment experiments. Protein was isolated from the sample

using the previously mentioned steps for protein isolation and quantification. Twenty

micrograms of proteins were separated by molecular weight via sodium dodecyl

polyacrylamide gel electrophoresis (SDS-PAGE) on a 12% polyacrylamide gel (37.5:1

Acrylamide/Bis) (MiniProtean II apparatus; Bio-Rad, Hercules, CA). After size

fractionation, proteins were transferred to polyvinylidene difluoride membranes (PVDF)

(Santa Cruz Biotech). The proteins were immunoblotted using an HC-3 specific rabbit

polyclonal antibody (0.11 μg/mL) (Mutyam et al., 2011), or mouse-anti-Beta-actin

antibody diluted 1:5,000 (Sigma-Aldrich) (Goldstein et al., 2010). Overnight at 4°C, the

membrane was then incubated with a goat anti-rabbit horseradish peroxidase-conjugated

secondary antibody diluted 1:1,000 (Santa Cruz Biotech, CA), or with a goat anti-mouse

horseradish peroxidase-conjugated secondary antibody diluted 1:1,000 (Santa Cruz

Biotech). A chemiluminesence substrate (West Pico SuperSignal, Pierce, Rockford, IL)

was used to detect immunofluorescence which was visualized on X-ray film (Kodak

Film, Rochester, NY).

Cell Fixation

At select time points throughout the study, 400 μL (~4 x 10^6 cells) were collected from culture and fixed for 20 minutes in 1mL periodate-lysine-paraformaldehyde fixative solution (500 μL 8% paraformaldehyde, 400uL lysine HCL,100 μL 0.1M phosphate buffer (1.37g sodium monobasic phosphate, 5.6g sodium dibasic phosphate, pH 7.2), and 0.001g sodium periodate). After fixing, the cells were centrifuged at 1,000 x g for 10 minutes and fixative supernatant was removed. Cells were resuspended in 1xPBS and placed on a 2% gelatin-coated slide. A 1 x 1 inch coverslip was used to smear the cells along the slide and remained at room temperature to dry.

Immunocytochemistry

Slides, with previously fixed and smeared erythrocytes, were submerged in 0.2% Triton X-100 to permeabilize the cells, followed by submersion in 1% glycine (3.00 g glycine in 300 mL 1X PBS) and 0.1% sodium borohydride (300mg sodium borohydrate in 300 mL 1X PBS) solutions to diminish excess paraformaldehyde which remained on the sample slides. 1% blocking serum (1: 10 dilution of 10% blocking serum: 10% goat serum, 4% bovine serum albumin, and 0.05% Tween-20) was used to block the slides for 1 hour, followed by exposure to 2.0 μg of HC-3 primary antibody in 10mL of 1% blocking serum overnight at 4°C. Slides were then washed with 1XPBS and exposed to goat anti-rabbit fluorescein-conjugated secondary antibody (Vector Laboratories, Inc., Burlingame, CA) diluted 1:1,000 in 1% blocking serum for 1 hour at room temperature. After the secondary antibody incubation, 80 μL of To-Pro/Vectasheild mix (1:500 To-Pro: Life Technologies, Grand Island, NY) in Vectashield Mounting Medium (Vector

Laboratories, Inc.) was added to each slide. The presence of HC-3 was identified via fluorescent microscopy using an Olympus Fluoview 1000 Laser Scanning Confocal Microscope with the following settings for Fitc: HV (755), gain (1.375), and offset (12) and Cy5: HV (490), gain (1), and offset (12).

Data Analysis

Data analysis of Western blotting was completed using densitometry via ImageJ software. The immunoreactive bands were normalized to β-actin and expressed as a percentage of the control treatments (set at 100%). Immunocytochemistry data was also analyzed by ImageJ software. Immunocytochemistry images were scanned with ImageJ to quantify the fluorescent intensity of HC-3 throughout each target cell. Using the ImageJ software, a line segment approximately 20 μm in length was drawn through the short axis of the erythrocyte images, and the profile of the relative fluorescent intensity was generated and plotted.

IV.   Results

HC-3 localization in H. chrysoscelis erythrocytes exposed to 1 μm cAMP and 1 μm

epinephrine

Immunocytochemistry was performed on *H. chrysoscelis* erythrocyte cultures

exposed to 1 μM cAMP, a second messenger, or 1 μM epinephrine in order to observe

the effect on HC-3 subcellular localization within erythrocytes. As shown previously,

immunocytochemistry confirmed that 48 hour control erythrocytes exhibit HC-3

distributed throughout the cytoplasm as indicated by a fluorescent signal dispersed

throughout the cytoplasm (Fig. 1B,E). In addition, immunocytochemistry of erythrocytes

cultured in CCCM and glycerol for 48 hours revealed fluorescent intensity specific to the

cell membrane which is indicative of HC-3 membrane localization (Fig. 1 C,F).

Furthermore, erythrocytes cultured in CCCM for 48 hours and exposed to cAMP for 30

minutes (Fig. 1 G,J) and 60 minutes (Fig. 1 H,K) showed an absence of HC-3 membrane

localization as HC-3 remained distributed in intracellular vessicles. However, fluorescent

intensity analysis of cross section of cells from erythrocytes exposed to cAMP for 90

minutes exhibit two fluorescently intense peaks which are localized to the cell membrane

(Fig. 1 I,L). In addition, fluorescent analysis of erythrocytes exposed to cAMP for 90

minutes also revealed a third fluorescent peak indicating HC-3 was still abundant in the

cytoplasm (Fig. 1 I,L). Cells cultured in CCCM and treated with 1 μM epinephrine for 30

minutes (Fig. 1 M,O) and 60 minutes (Fig. 1 N,Q) exhibited prominent membrane

localization when compared to 48 hour control cells. Immunocytochemistry analysis of

cells treated with epinephrine showed intense peaks of fluorescent intensity which is

absent in control erythrocytes (Fig. 1 O,Q). Fluorescent analysis results from erythrocytes

treated with epinephrine for 30 minutes (Fig. 1 M,O) show an additional fluorescently intense peak indicating HC-3 still remains in the cytoplasm which suggests that HC-3 has begun to be translocated to the membrane. Furthermore, erythrocytes treated with epinephrine for 60 minutes have enhanced HC-3 membrane localization and reduced cytoplasmic fluorescent intensity as a result of greater HC-3 membrane trafficking (Fig. 1 N,Q). In addition, it was also observed in the 60 minute epinephrine treatment group that cells exhibited increased fluorescence throughout the entire cell, potentially masking HC-3 localized fluorescent intensity in the membrane which suggests possible HC-3 upregulation (Fig. 1 N,P). Lastly, preincubation of the primary antibody with the immunoreactive HC-3 peptide eliminated much of the fluorescent signal, indicating the fluorescence observed is HC-3 specific (Fig. 1 A,D).
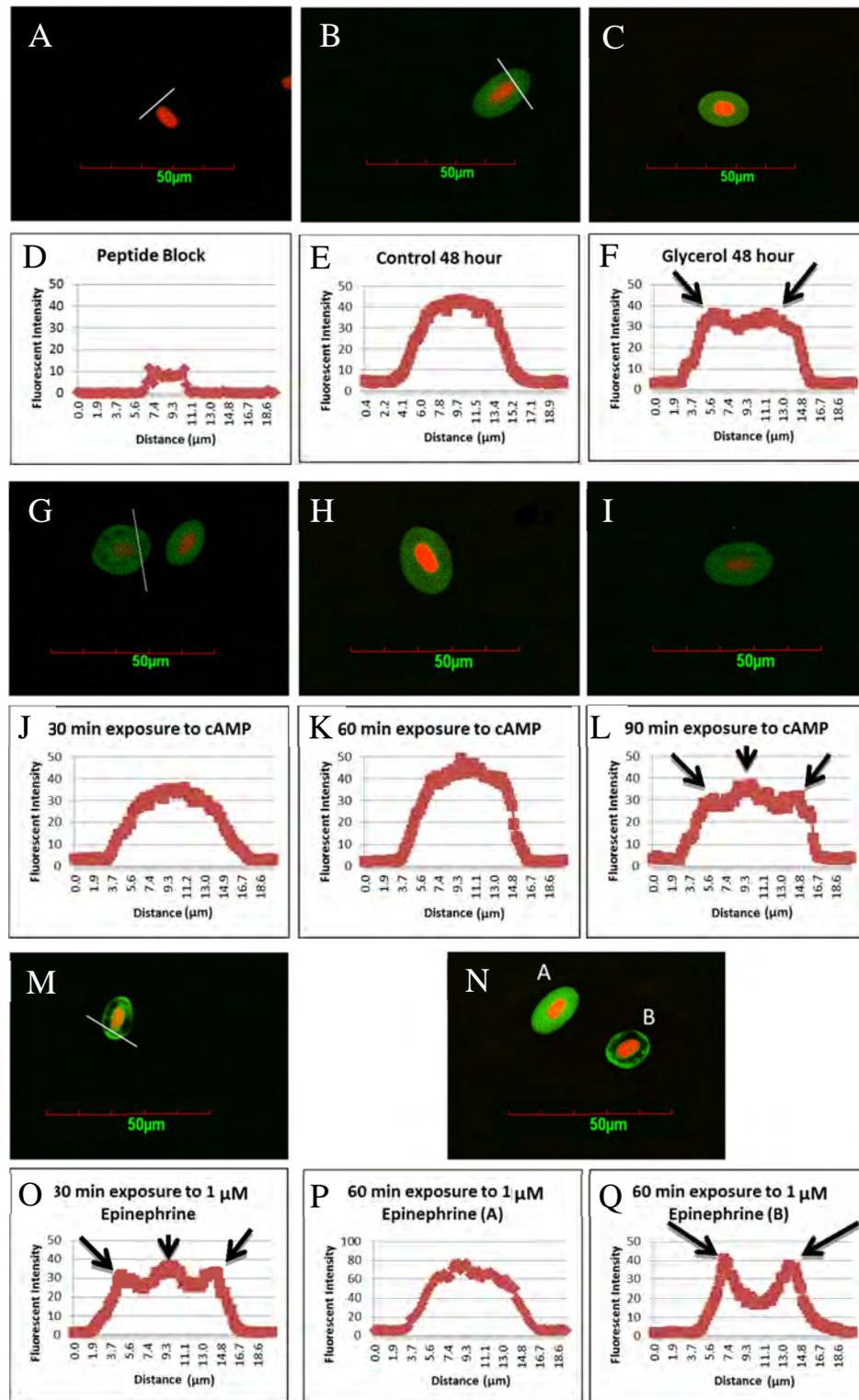
Figure 1. HC-3 subcellular localization in cultured *H. chrysoscelis* red blood cells exposed to cAMP and epinephrine.

Cells cultured in CCCM (A), CCCM with 156mM glycerol (B), and cells in CCCM exposed to cAMP (G-I), or epinephrine (M-N) were fixed and fluorescent immunocytochemistry using a primary antibody directed against HC-3 and a fluorescein conjugated secondary antibody was used to examine HC-3 subcellular localization in erythrocytes (A-C,G-I, M-N). Expression of HC-3 (green) was visualized with confocal microscopy. Nuclei were counterstained with To-Pro (red). Semi-quantitative analysis of the fluorescent intensity of HC-3 immunofluorescence was measured via ImageJ software (D-F, J-L, O-Q). (n=20 cells per treatment).

HC-3 localization in H. chrysoscelis erythrocytes exposed to calphostin C, epinephrine,

or calphostin C+ epinephrine.

Data from cAMP or epinephrine treatment of *H. chrysoscelis* erythrocytes

performed earlier in this study revealed enhanced HC-3 membrane localization in cells

exposed to epinephrine. To further elucidate the mechanism of HC-3 trafficking in

erythrocytes, calphostin C, a PKC, inhibitor was used to inhibit PKC activity and observe

the effects on HC-3 membrane localization. Fluorescent analysis results from

erythrocytes cultured in CCCM and glycerol for 48 hours exhibited two fluorescently

intense peaks which indicates HC-3 localization at the membrane (Fig. 2 B,D).

Erythrocytes cultured in CCCM for 48 hours then treated with 1 μM calphostin C for 60

minutes exhibited similar HC-3 expression in intracellular vesicles throughout the

cytoplasm when compared to 48 hour control cells (Fig. 2 E,H). In addition, erythrocytes

cultured in CCCM for 48 hours then administered 1 μM epinephrine (Fig. 2 F,I) exhibited

a similar increase in fluorescent intensity throughout the entire cell as observed earlier in

this study (Fig. 1 N,P). The increased fluorescent intensity observed in these cells could

possibly be attributed to upregulation of the HC-3 protein, which could potentially mask

membrane-specific fluorescence as a result of HC-3 trafficking. Furthermore, cells

administered the calphostin C pre-treatment followed by epinephrine (Fig. 2 G,I)

exhibited HC-3 localization restricted to the cytoplasm similar to control cells.
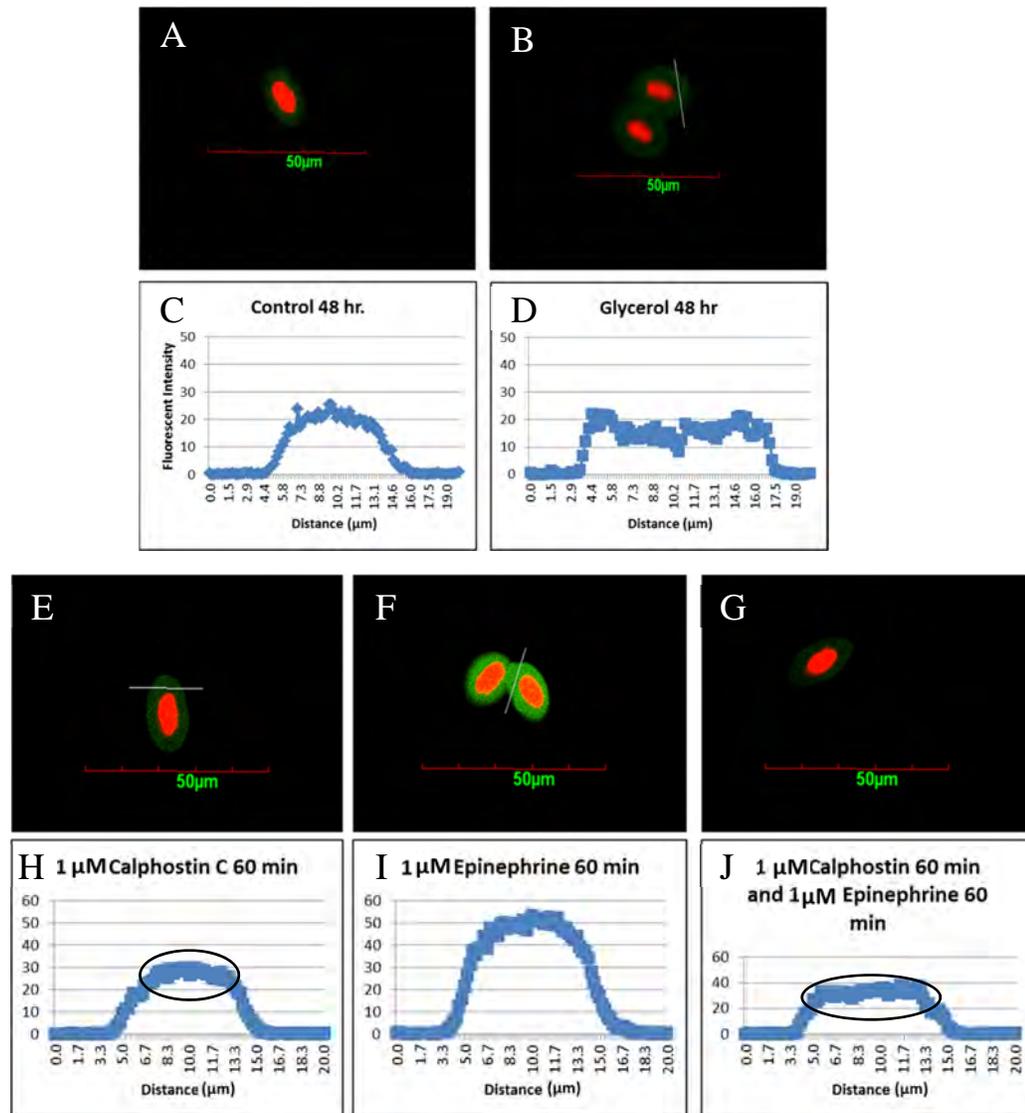
Figure 2. HC-3 subcellular localization in cultured *H. chrysoscelis*

erythrocytes exposed to epinephrine and PKC inhibitor calphostin C.

Fluorescent immunocytochemistry was used to examine HC-3 subcellular

localization in *H. chrysoscelis* erythrocytes. Fluorescent intensity was measured

via ImageJ software (C-D, H-J). Erythrocytes cultured in CCCM (A), CCCM with

156mM glycerol (B), and cells in CCCM exposed to calphostin C (E),

epinephrine (F), and preincubation of calphostin C followed by administration of

epinephrine (G) were fixed on 2% gelatinized slides and blocked with 1% goat

serum. A 1:1000 dilution of HC-3 primary rabbit antibody was administered

overnight followed by exposure of secondary antibody tagged with fluorescein

(1:1000). Expression of HC-3 (green) was visualized with confocal microscopy.

Nuclei were counterstained with To-Pro (red). (n=20 cells per treatment).

Native and glycosylated HC-3 abundance in H. chrysoscelis erythrocytes exposed to calphostin C, epinephrine, or calphostin C+ epinephrine.

It is hypothesized that native HC-3 is present in intracellular vesicles throughout the cytoplasm, and native HC-3 which has been post-translationally modified via glycosylation is found localized in the cell membrane (Mutyam et al. 2011). Immunocytochemistry results in this study cultured erythrocytes exposed to epinephrine enhanced HC-3 membrane localization, while calphostin C prevented this event. To determine the relative distribution of glycosylated and native HC-3 protein expression in erythrocytes, Western blotting performed on erythrocyte proteins (Fig. 3A). Western blot analysis of proteins isolated from erythrocytes cultured in CCCM and glycerol for 48 hours exhibited increased glycosylated and native HC-3 expression when compared to control cells (Fig. 3B). As expected, cells cultured in CCCM and exposed to 1 μM calphostin C for 60 minutes displayed similar expression levels of glycosylated and native HC-3 as the control treatment (Fig. 3B). However, erythrocytes cultured in CCCM for 48 hours then treated with 1 μM epinephrine for 60 minutes displayed a 50% increase in glycosylated HC-3 expression and a 2 –fold increase in native HC-3 expression when compared to control erythrocytes (Fig. 3B). Erythrocytes preincubated with 1 μM calphostin C for 60 minutes followed by incubation with 1 μM epinephrine for 60 minutes were similar to control cells (Fig. 3B). Lastly, erythrocytes preincubated with calphostin C prior to epinephrine exposure exhibited a 2-fold increase of glycosylated HC-3 expression as compared to control cells (Fig. 3B).
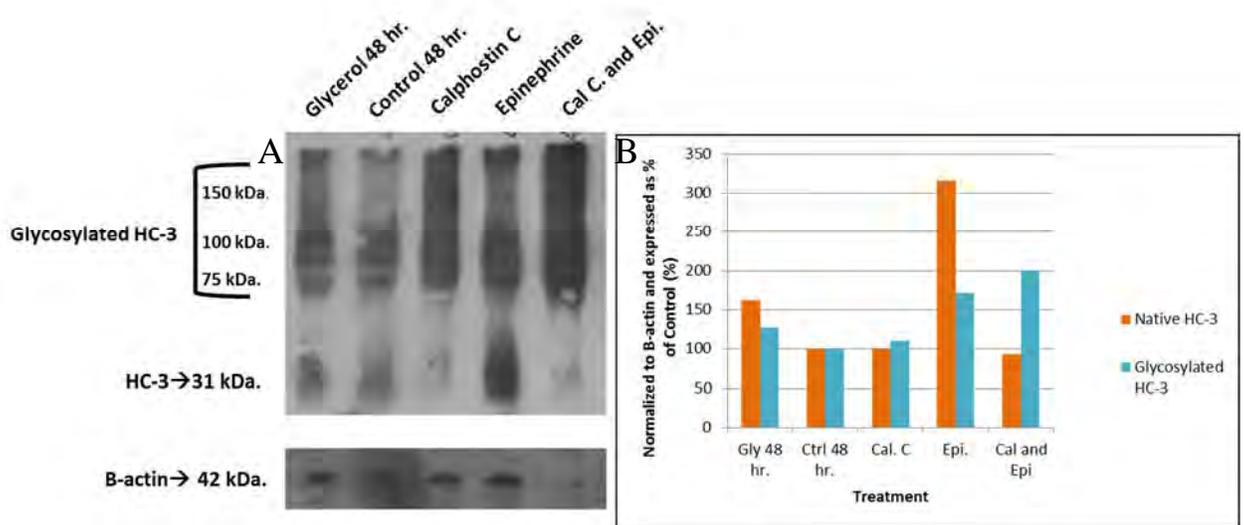
Figure 3. HC-3 protein expression in cultured *H. chrysoscelis* erythrocytes exposed to epinephrine and PKC inhibitor calphostin C.

Western blotting was used to quantify the expression native and glycosylated HC-3 protein in erythrocytes cultured in CCCM and CCCM with 150 mM glycerol, and cells in CCCM exposed to calphostin C, epinephrine, and preincubation with calphostin C followed by treatment with epinephrine (A). Densitometry was performed via ImageJ software to compare the expression levels of native (31 kDa.) and glycosylated HC-3 (75-150 kDa.) in each treatment (B). Values were normalized to β-actin, and expressed as a percentage of the control treatment.

Phosphorylation at Threonine 514 and Serine 660 residue sites on PKC in cultured H. chrysoscelis erythrocytes exposed to calphostin C, epinephrine, or calphostin C+ epinephrine

Calphostin C is a PKC inhibitor that binds to the regulatory domain of active and inactive PKC thereby inhibiting PKC activity by preventing diacyl glycerol (DAG) from binding to PKC and activating it. In order to assess how PKC activity parallels the HC-3 membrane translocation induced by epinephrine, a Western blot was performed on erythrocyte proteins to analyze phosphorylation at Thr. (514) (Fig. 4 A) and Ser. (660) (Fig. 4 B) amino acid sites in PKC where phosphorylation can occur. Thr. (514) amino acid residue in PKC was found to be phosphorylated in the control, glycerol, 1 μM epinephrine, and 1 μM calphostin C and 1 μM epinephrine treatment groups at a similar level without specificity for a particular treatment (Fig. 4 C). However, in the 1 μM calphostin C treated erythrocytes, the expression of phosphorylated PKC at the Thr. (514) residue was approximately 30% less than the control cells which was expected since calphostin C would reduce PKC activity. Moreover, expression of phosphorylation at the Ser. (660) in PKC in cells cultured in CCCM with glycerol for 48 hours were 20% lower than that of the control cells (Fig. 4D). Similar to the reduced phosphorylated Thr. (514) level in the calphostin C treatment (Fig. 4C), Ser. (660) phosphorylated levels were 20% lower than that of control cells which was also expected (Fig. 4 D). In addition, erythrocytes exposed to epinephrine (Fig. 4D), exhibited a 10% increase in phosphorylation at the Ser. (660) site that was expected as epinephrine leads to PKC activation. However, the calphostin C and epinephrine treatment (Fig. 4D) also showed a

10% increase of phosphorylation at the Ser. (660) residue which was not expected due to the presence of the inhibitor.
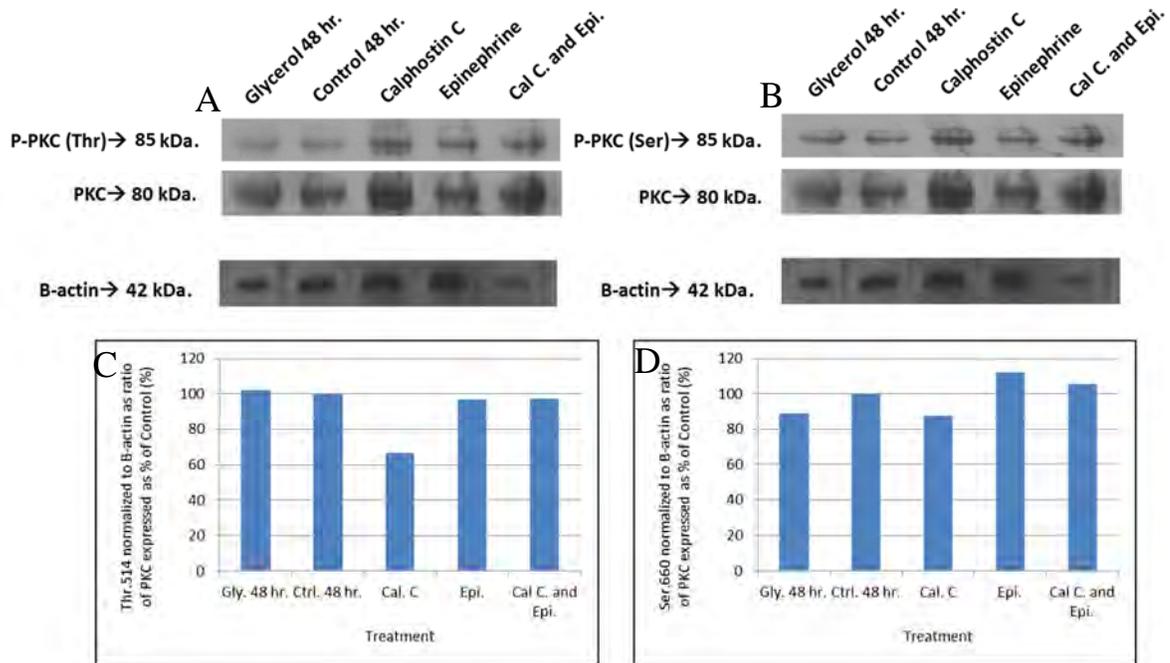
Figure 4. Phosphorylation of PKC at threonine (514) and serine (660) amino acid residues in cultured *H. chrysoscelis* erythrocyte exposed to epinephrine and calphostin C.

Western blotting was used to quantify the expression PKC (80 kDa.) and phosphorylated PKC (85 kDa.) at a threonine (514) amino acid (A) and a serine (660) (B) in erythrocytes cultured in CCCM and CCCM with 150 mM glycerol, and cells in CCCM treated with calphostin C, epinephrine, or preincubation with calphostin C followed by epinephrine. Densitometry was used to analyze levels of PKC and phosphor-PKC at the threonine (C) and serine (D) residues. Values were normalized to β-actin (42 kDa.), and the ratio of P-PKC/PKC was expressed as a percentage of the control.

V.   Discussion

Maintaining proper fluid homeostasis is essential for freeze tolerant organisms to survive freezing and thawing. When extracellular fluid begins to freeze as external changes occur in the environment, a hyperosmotic environment is created which causes rapid water efflux from a cell. In contrast, during the spring season when temperatures rise, ice in the extracellular fluid region thaws creating a hypoosmotic environment resulting in rapid water influx into a cell (Mazure, 1988). *Hyla chrysoscelis* regulates water movement between the extracellular fluid and the treefrog's cells through the use of glycerol as a cryoprotectant (Storey and Storey, 1992; Storey, 1997). It has been previously hypothesized that an aquaglyceroporin found in *H. chrysoscelis,* HC-3, contributes to the freeze tolerance of Cope's gray treefrog by increasing cell membrane permeability of glycerol (Zimmerman et al., 2007). Mutyam et al. (2011) contributed to this hypothesis when it was observed that erythrocytes isolated from cold-acclimated treefrogs have significant amounts of HC-3 proteins localized within the cell membrane when compared erythrocytes compared to warm-acclimated treefrogs. Mutyam et al. (2011) also suggested that HC-3 remains in intracellular vesicles prior to a physiological or cellular trigger that cues vesicular trafficking and HC-3 membrane localization.

Furthermore, we hypothesize the HC-3 translocation event from the cytoplasm to the cell membrane is initiated by the neuroendocrine hormone, epinephrine, which acts through second-messenger pathways in order to regulate HC-3 expression as part of the mechanism of freeze tolerance demonstrated in *H. chrysoscelis.* In the present study, *H. chrysoscelis* erythrocyte cultures were exposed to soluble cAMP, a second messenger, or

epinephrine to mimic the *in vivo* environment to order to observe the effects on HC-3

subcellular localization and expression within the red blood cells. In this study,

erythrocytes cultured in CCCM with 150mM glycerol for 48 hours exhibited enhanced

HC-3 membrane localization, while control cells cultured in CCCM for 48 hours were

observed to have HC-3 remain in intracellular vesicles within the cytoplasm, consistent

with prior results (Mutyam et al., 2011). In addition, the results of immunocytochemistry

of erythrocytes treated with epinephrine suggest that epinephrine is one agonist which

functions to regulate HC-3 expression within red blood cells. This study shows that

within 30 minutes of epinephrine exposure, the mechanism for HC-3 membrane

localization has already been initiated. Moreover, at 60 minutes into the exposure time,

erythrocytes show increased HC-3 membrane localization with reduced intracellular

vesicle distribution. Lastly, results from this study show 90 minutes after exposure to

soluble cAMP, *H. chrysoscelis* erythrocytes began membrane translocation of HC-3.

Our results are consistent with the results of mammalian membrane trafficking

studies focused on the mammalian ortholog of HC-3: AQP3. In addition, Caco-2 cells,

epinephrine was shown to induce AQP3 trafficking in cells exposed to epinephrine via a

PKC-dependent pathway (Yasui et al., 2008). Furthermore, exposure of these Caco-2

cells to epinephrine also led to the increase of AQP3 mRNA levels which suggests

epinephrine may also be involved in AQP3 transcriptional upregulation (Asai et al.,

2006). It has been previously reported that erythrocytes isolated from cold-acclimated

treefrogs display native HC-3 at 31.5 kDa., as well as an increase in a high molecular

weight species > 65-150 kDa. which is hypothesized to represent glycosylated HC-3

within the cell membrane (Pandey et al., 2010; Mutyam et al., 2011). Similarly, results from this study show cultured *H. chrysoscelis* erythrocytes that were exposed to epinephrine exhibited a 3–fold increase in native HC-3 expression which was absent in other treatment groups. Therefore, HC-3 in *H. chrysoscelis* erythrocytes may follow a similar mechanism of epinephrine-induced AQP3 upregulation in Caco-2 cells as noted by Asai et al. (2006). In addition, the erythrocytes exposed to epinephrine also showed an enhanced expression of glycosylated HC-3, which further suggests a mechanism for epinephrine-induced HC-3 membrane localization.

Further investigation of the epinephrine-induced mechanism of HC-3 translocation by using calphostin C, a PKC inhibitor that inhibits PKC activity, indicates PKC activity for HC-3 translocation from the cytoplasm to the membrane via epinephrine initiation. Erythrocytes preincubated with calphostin C followed with epinephrine administration showed no enhanced membrane localization similar to control cells. Therefore, these data suggest that upon epinephrine binding to a G protein coupled receptor (GpCR), a PKC-dependent mechanism is initiated resulting in HC-3 membrane localization. These results parallel findings from Yasui et al. (2008) who concluded APQ3 subcellular localization is regulated by a PKC-dependent mechanism in mammalian Caco-2 cells. Furthermore, the inhibition of PKC results in low native HC-3 expression level similar to that of control cells, while epinephrine treated cells exhibited a 3 –fold increase in native HC-3. This suggests another possible downstream effect of epinephrine in *H. chrysoscelis* erythrocytes is HC-3 upregulation via a PKC-dependent mechanism.

Also, investigation of phosphorylated PKC at Thr. (514) and Ser. (660) residues may indicate *H. chrysoscelis* erythrocytes utilize two different mechanisms for HC-3 membrane localization. The first mechanism, which we propose is a PKC-dependent initiated by epinephrine, while the second mechanism is mediated by glycerol. In this study, increased levels of phosphorylated PKC at the Ser. (660) residue were found only in erythrocyte cultures where cells were exposed to epinephrine and not glycerol. In addition, we hypothesize that PKC is necessary for vesicular trafficking of HC-3 to the membrane in the presence of epinephrine. However, cells cultured in CCCM with 150mM glycerol also display membrane localization of the aquaglyceroporin, and did not exhibit increased PKC phosphorylation at either the Thr. (514) or Ser. (660) residue. This suggests that glycerol mediates HC-3 membrane trafficking through a separate mechanism that does not employ PKC.

In conclusion, we propose *H. chrysoscelis* erythrocytes utilize an epinephrine-induced PKC-dependent pathway to translocate HC-3 to the membrane during cold-acclimation as part of the organism's freeze-tolerant mechanism required for survival in freezing and sub-freezing temperatures. Understanding the vesicular trafficking mechanism of aquaglyceroporin HC-3 in *H. chrysoscelis* is important for biomedical applications. If a better understanding of the mechanism by which *H. chrysoscelis* cryoprotects itself, the data can be useful in organ transplant strategies for humans. Subsequent findings will aid in human organ preservation by increasing viability in transplants by inducing a similar mechanism in mammals through AQP3.

VI.   Future Directions

In order to further investigate the mechanism of HC-3 membrane translocation in *H. chrysoscelis* erythrocytes, upcoming work needs to be conducted into the signaling pathways initiated by epinephrine. This study reveals that epinephrine initiates a PKC-dependent mechanism that is responsible for regulating HC-3 protein expression and subcellular localization. Further experiments should focus on activating PKC to test the hypothesis that activating PKC will enhance HC-3 membrane translocation and upregulation of HC-3.

Additionally, results of this study suggest that in *H. chrysoscelis* erythrocytes, a glycerol mediated pathway and an epinephrine pathway are both responsible for HC-3 membrane translocation. Perhaps erythrocytes cultured in CCCM for 150mM glycerol for 48 hours and administered epinephrine for 60, 90, and 120 minutes could induce a synergistic effect with enhanced HC-3 membrane localization and possible HC-3 upregulation.

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