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Upstream regulators of VRAC activation in Human 1321N1 Astrocytoma Cells

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UPSTREAM REGULATORS OF VRAC ACTIVATION IN HUMAN 1321N1 ASTROCYTOMA CELLS

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By

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I HEREBY RECOMMEND THAT THE THESIS PREPARED UNDER MY SUPERVISION BY Courtney Elyse Moore ENTITLED Upstream regulators of VRAC activation in Human 1321N1 Astrocytoma Cells BE ACCEPTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF Master of Science

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Abstract

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Cells rely on a constant volume not only for structural stability but also for normal homeostatic processes to occur. In the brain and CNS, cells can regain their normal volume through a process termed regulatory volume decrease (RVD). A key component of a cells' response to cell swelling is the activation of channel(s) responsible for the efflux of chloride current, $I_{Cl,swell}$. Volume regulated anion channels (VRAC) which mediate $I_{Cl,swell}$ have been implicated in controlling cell volume during RVD, but the mechanisms which activate this channel are not completely understood. In this study, I examined the role of G protein-coupled signaling via P2Y2 purinergic receptors for activation of VRAC in osmotically swollen human astrocytoma cells. Whole cell patch clamp recordings were performed on 1321N1 cells stably transfected to express the human P2Y2 receptor. This tumor cell does not show VRAC activation in the native cell type; however, VRAC activation is displayed in the transfected 1321N1 cells. Cells were sequentially perfused with isoosmotic (290 mOsm) and hypoosmotic (200 mOsm and 250 mOsm) solutions containing 100 mM CsCl as the major electrolyte. The same CsCl concentration was used in the isoosmotic patch electrode solution. Voltage clamp recordings lasting 100 msec were made in 20 mV steps between -100 mV and +120 mV

every 30 sec. For some experiments, inhibitors and activators of GPCRs, purinergic signaling, and ATP release pathways were added to the perfusate solutions. In others, pertussis toxin was added to the patch electrode solution. Hypoosmotic exposure evoked an outwardly rectifying current which inactivated over time at the higher membrane voltages and was inhibited by DCPIB; characteristics ascribed to VRAC. A smaller VRAC current was observed when cells were perfused with a 250 mOsm hypoosmotic CsCl solution. VRAC was not activated in isoosmotic solutions but the VRAC response in hypoosmotic solutions was enhanced in the presence of thrombin. VRAC activation was completely blocked by adding suramin to the hypoosmotic solution or pertussis toxin to the patch electrode solution, both of which are inhibitors of G-protein signaling pathways. Carbenoxolone, which can block ATP release from swollen cells and can directly inhibit VRAC also blocked VRAC activation. Adding ATP did not rescue cells from this inhibition; however, adding thrombin to activate a separate G-protein coupled signaling pathway did result in a partial recovery. In contrast, meclofenamate, another ATP efflux inhibitor had no effect on the VRAC response to hypoosmotic exposure. I conclude GPCRs are necessary for VRAC activation in these human astrocytoma cells. Exogenous thrombin increased an osmotically-sensitive current but, like exogenous ATP, had no effect on VRAC activation during isoosmotic exposure. If endogenously released ATP is responsible for VRAC activation via the P2Y2 receptor, it is not effluxed via gap junction hemi-channels. Future studies are needed to measure ATP release during hypoosmotic exposure and identify the specific GPCR that mediates VRAC activation.

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A. Cell Volume Regulation

Introduction

Cells rely on a constant volume not only for structural stability but also for normal homeostatic processes to occur including cell proliferation, apoptosis, and migration (Okada, Sato & Numata 2009). Most mammalian cells are bathed in extracellular fluid at a constant osmolarity however injury or stress can lead to changes in the extracellular osmolarity and cell volume alterations. These alterations in cell volume may be limited by extracellular tissue constraints. For example, brain volume is limited by the size of the cranium. Considering this limitation of brain tissue, the ability of cells to regulate their volume through osmolyte movement via uptake, release, and/or breakdown, is critical for cell survival (Lang et al. 1998).

Two major types of cells are in the brain parenchyma: neurons and glial cells. Neurons, the basic functional unit of the nervous system, are electrically excitable cells with specialized structures and biochemistry for conducting nerve impulses and communicating with other cells in the central nervous system (Purves et al. 2001). Astrocytes are a type of glial cells and are the largest and most numerous cells in the gray matter of brain and spinal cord. These cells regulate the extracellular ionic and chemical environment and have been implicated in modifying neuronal signaling (Chung, Allen & Eroglu 2015). In the 1960s, astrocytes were considered to do little more than occupy

space in the CNS (Van Herreveld, Crowell & Malhorta 1965). However since then, scientists have found that astrocytes play an important role in brain function by acting not only through extracellular buffering of neurotransmitters but also through cellular signaling and volume regulation. Depending on location in the brain, astrocytes occupy approximately 20-30% of the brain volume (Nedergaard, Ransom & Goldman 2003) and are well known for regulating the extracellular space around neurons (Chung, Allen & Eroglu 2015). Astrocytes have also been shown to protect neurons and increase the neuron cell survival rate in many pathological settings (Chen & Swanson 2003). Manipulating astrocyte function is thus an important strategy to enhance neuronal survival and improve the outcome following brain injury.

Regulating cell volume

Cell volume is regulated through various mechanisms at all times. In addition to volume regulatory mechanisms that are active during osmotic stress, cells also regulate volume under homeostatic conditions. Because there is a constant passive flow of ions and metabolites into and out of cells, cell volume control mechanisms are active for homeostasis or steady-state conditions. Flow of solutes and water across a cell's semi-permeable plasma membrane is driven by electrochemical gradients and osmotic pressure, respectively. In an ideal semi-permeable membrane at equilibrium, solute concentrations would be equalized across the divide; however in mammalian cells, an electrochemical gradient is present for most inorganic ions and organic osmolytes. The cell maintains a constant cell volume by active and passive solute transport across the cell membrane. Under steady-state conditions, this passive osmolyte flow into and out of the

cell consists mainly of potassium, sodium, and chloride ions. To balance this passive flow of osmolytes driven by their electrochemical gradient, mammalian cells possess a sodium-potassium enzyme (Na^+/K^+-ATPase) found on the plasma membrane which pumps positively-charged sodium ions out of the cell and positively-charged potassium ions into the cell against their concentration gradients.

During cell shrinkage (i.e. hypertonic stress), many cells respond with active uptake of organic osmolytes and inorganic ions through various mechanisms. This uptake is coupled to an influx of water and is termed regulatory volume increase (RVI). RVI uptake mechanisms include the exchange of hydrogen and sodium ions via the Na^-/H^+ antiporter, the exchange of chloride and bicarbonate through the $Cl^-/HCO3^-$ antiporter, the uptake of sodium, potassium and chloride ions through the NKCC symporter, and the uptake of sodium chloride through the Na^+/Cl^- symporter (Mongin & Orlov 2001). Initial RVI responses to acute cell shrinkage results in the net accumulation of inorganic ions such as sodium, potassium, bicarbonate, and chloride. But, the solutes accumulating during prolonged cell shrinkage also consist of organic osmolytes such as sorbitol, taurine, and betaine (Hoffman, Lambert & Pedersen 2009, Wehner et al. 2003).

In contrast, after acute swelling, cells undergo regulatory volume decrease (RVD) by releasing ions and organic osmolytes. RVD involves the release of inorganic ions and organic osmolytes (Lang et al. 1998). This efflux is accomplished mainly through potassium and chloride channels or transporters but it is also achieved through the extrusion of amino acids such as taurine and polyols (Pasantes-Morales, Murray, Sanchez-Olea & Morán 1994, Pasantes-Morales et al. 2000, Ordaz et al. 2004).

B. Chloride Channels

Chloride is the most abundant anion in most mammalian cells under physiological conditions. Chloride movement into and out of cells occurs through various active and passive transport pathways. The energetic cost for chloride ion movement is relatively low in most mammalian cells as the equilibrium potential for chloride is close to the normal plasma membrane resting potential. The normal reversal potential for chloride in neurons is approximately -60 mV (Purves et al. 2001). However, astrocytes are high chloride cells. Cultured rat astrocytes demonstrate internal chloride concentrations between 20 and 40 mM (Kettenmann, Backus & Schachner 1987). As a result, rat astrocytes display an equilibrium potential for chloride of -31 mV (Lascola & Kraig 1996). In addition, chloride movement also occurs when a positively-charged cation, such as potassium, is effluxed. Thus the net movement of these ions is electrically neutral. Passive chloride efflux requires that the equilibrium potential for Cl^- is more positive than the cell's membrane potential. During homeostatic, or steady-state, conditions, chloride movement is achieved through transporters such as the Na^+/Cl^- cotransporter, $Na^+/K^+/2Cl^-$ cotransporter (NKCC), Cl^-/HCO_3^- anion exchanger, and the K^+/Cl^- cotransporter or through various anion channels including Ca^{2+}-activated Cl^- channels (CaCC), cAMP/PKA-activated cystic fibrosis transmembrane conductor regulator (CFTR), members of the chloride channel family (ClC-*X*), and volume-regulated anion channels (VRAC) (Hoffman, Lambert & Pedersen 2009). In cells with a small electrochemical gradient of chloride, chloride movement is minimal during steady-state conditions. With active transport, such as during RVI, there is secondarily active uptake

of chloride that occurs via the NaCl symporter and the NKCC symporter (Hoffman, Lambert & Pedersen 2009, Wehner et al. 2003). In contrast, chloride is passively and actively transported to the extracellular fluid during RVD through VRAC, ClC-*X*, and the K^+/Cl^- cotransporter (Hoffman, Lambert & Pedersen 2009). Chloride movement has been extensively studied in transepithelial transport in the kidney (Lang et al. 1990, Lang & Paulmichl 1995, Furst et al. 2000, Helix, Strobaek, Dahl & Christophersen 2003) as well as the intestine and endothelial cells (Kubo & Okada 1992, Nilius, Eggermont & Droogmans 2000, Lim et al. 2006); however less information is known about chloride movement in neurons and glial cells in the CNS.

Chloride channels implicated in volume regulation

Because chloride is the most abundant anion in cells, chloride can be effective as an osmolyte controlling water movement and cell volume. In astrocytes, organic osmolytes and Cl^- fluxes contribute to cell volume regulation in response to acute and chronic osmotic challenges (Mongin & Kimelberg 2002). Chloride is released from cells during acute cell swelling through channels and transporters on the plasma membrane. It has been estimated that 60-70% of chloride efflux following acute cell swelling occurs through channels in cultured astrocytes and human glioma cells (Ernest, Weaver, Van Duyn & Sontheimer 2005).

Electrophysiology of cells during RVD due to chloride conductance

Chloride current is minimal under steady state conditions; however, chloride conductance has been found to increase during hypoosmotic swelling (Bakhramov, Fenech & Bolton 1995) but not in hypertonically shrunken cells (Estevez, Bond & Strange 2001). The chloride current activated during swelling, $I_{Cl,swell}$, is ubiquitously present in mammalian cells (Duan et al. 1997, Jentsch 2016).

$I_{Cl,swell}$, had long been characterized by its electrophysiological behavior; however the identity of the specific molecule responsible for $I_{Cl,swell}$ was unknown until more recently. This current activates at positive potentials under hypoosmotic conditions and thus, is outwardly rectifying. In HEK293 cells, $I_{Cl,swell}$ is not dependent on changes in intracellular calcium concentration (Weylandt et al. 2003). Two voltage-gated chloride channels have been implicated in volume decrease following osmotic swelling, ClC-2 and ClC-3. ClC-2 channels generate an inwardly rectifying current and are inhibited by chloride channel blockers and cadmium ions. ClC-3 is an outwardly rectifying channel which is assumed to be constitutively open when present in the cell membrane. Until recently, the ClC-3 channel protein had been proposed as the prominent protein that encodes the channel responsible for $I_{Cl,swell}$, but more recent studies have suggested otherwise (Duan et al. 1997, Weylandt et al. 2003, Syeda et al. 2016). VRAC is characterized by an outwardly rectifying anion-selective current at depolarized potentials (Blum, Walsh & Dubyak 2009). The current that is associated with VRAC develops within the first few minutes following hypoosmotic challenge. Inactivation of this current occurs at more positive potentials and the degree of this inactivation has been found to vary between cell types (Nilius, Sehrer & Droogmans 1994). VRAC also is permeable to

some organic osmolytes (Mongin & Kimelberg 2002) such as taurine and glutamate and this channel has been shown to be a major pathway for excitatory amino acid release (Basarsky, Feighan & MacVicar 1999, Kimelberg & Mongin 1998, Phillis, Song & O'Regan 1997).

Volume regulated anion channels

In the brain, volume regulated anion channels, VRACs, are widely expressed and have been proposed to play an important role not only in cell proliferation, apoptosis, swelling-induced exocytosis and control of cell membrane potential (Okada et al. 2001, Nilius & Droogmans 2003). These channels have also been shown to play an important role in preventing detrimental cellular swelling, apoptosis and cell death during cerebral ischemia (Mongin 2007, Adler 2014).

Research performed by Ernest and colleagues on cultured astrocytes and human glioma cells found the inhibition of swelling-induced chloride channels and transporters also inhibits RVD. These findings suggest that the volume increase is limited by quickly activating volume-regulatory mechanisms (Ernest, Weaver, Van Duyn & Sontheimer 2005). Furthermore, volume-sensitive chloride current commonly associated with VRAC can be activated independently of osmotic volume changes (Manolopoulos, Prenen, Droogmans & Nilius 1997, Shimizu, Numata & Okada 2004). For example, electrophysiological recordings using calf pulmonary artery endothelial cells found that volume-sensitive chloride current may be activated by thrombin (Manolopoulos, Prenen, Droogmans & Nilius 1997). Other patch clamp studies using human cervix HeLa cells

have shown $I_{Cl,swell}$ can be activated or enhanced independently of volume through the application of GTPγS, TNFα, and ATP (Shimizu, Numata & Okada 2004).

Identification of LRRCs as components of VRAC

Although VRAC's molecular identity is still not entirely understood, recent studies have found that VRAC is formed by a heterooligomer of the leucine-rich-repeat-containing proteins (LRRC) (Stauber 2015). One of these proteins, LRRC8, was originally discovered and cataloged in 2003 and since then, there have been five isoforms of this protein labeled: LRRC8A-E. LRRC8A was first studied in its mutated, dysfunctional form which causes a rare type of agammaglobulinemia (Sawada et al. 2003). LRRC8A knockout mice also exhibited a dramatic reduction in excitatory amino acid release when exposed to hypoosmotic media (Hyzinski-Garcia, Rudkouskaya & Mongin 2014). Further studies have found that T cell development and function were negatively affected in LRRC8A knockout and these transgenic mice also demonstrated a modest block in B cell development (Kumar et al. 2014). No experiments to identify VRAC have been performed with the LRRC8A knockout mice *in vivo*; however, electrophysiological recordings using LRRC8A knockdown HEK cells showed a strongly suppressed $I_{Cl,swell}$ under hypoosmotic conditions (Voss et al. 2014).

In HeLa cells, LRRC8A proteins form an oligomer of up to four subunits, termed SWELL1, during osmotic challenges. The SWELL1 proteins then assemble to form complexes. These complexes are sufficient to form anion channels sensitive to osmotic stress (Syeda et al. 2016). Topology suggests the leucine-rich pore forming structure is

imbedded inside the plasma membrane in comparison to previous studies suggesting VRAC must be located on the outside of the plasma membrane to function properly during RVD (Qiu et al. 2014). Qui and colleagues concluded that SWELL1 forms a pore-like structure but it is unlikely that this protein functions on its own because over-expression of LRRC8A did not increase VRAC activity (Qiu et al. 2014). Channel gating and pore formation of SWELL1 during swelling is still not entirely understood.

Drugs used to identify chloride channels activated in RVD

A variety of channel blockers have been used to study the role of VRAC for RVD. This has permitted a characterization of the channel based on the selective ability of these drugs to inhibit its conductance. Pharmacologically, VRAC conductance is blocked partially or entirely by DIDS, SITS, NPPB, and DCPIB (Bowens, Dohare, Kuo & Mongin 2012, Hoffman, Lambert & Pedersen 2009). In murine CNS cells, application of DIDS (4,4'-diisothiocyanatostilbene-2,2'-disulphonic acid) in the extracellular fluid inhibited volume-sensitive chloride current through VRAC (Harvey, Saul, Garner & McDonald 2010). In other experiments using rat cerebral cortex slices, SITS and NPPB were applied to the extracellular media. SITS (4-acetamido-4'-isothiocyanostrilbene-2,2'-disulfonic acid) and NPPB (5-nitro-2-(3-phenyl-propylamino)benzoic acid) were found to also inhibit RVD through VRAC (Phillis, Song & O'Regan 1997, Shen, Wu & Chou 1996). These inhibitors also block other chloride channels. However, research performed by Decher and colleagues concluded that the ethacrynic acid derivative DCPIB (4-(2-butyl-6,7-dichlor-2-cyclopentylindan-1-on-5-yl) oxobutyric acid) is a more selective blocker of VRAC over other chloride channels (Decher et al. 2001). DCPIB inhibition is

reversible and not voltage dependent (Nilius & Droogmans 2003, Abdullaev et al. 2006). DCPIB blocks swelling induced chloride current *in vitro* in calf bovine pulmonary artery endothelial cells, guinea-pig atrial cardiomyocytes and *Xenopus* oocytes (Decher et al. 2001). In cultured astrocytes and human glioma cells, DCPIB treatment inhibited RVD and excitatory amino acid release (Ernest, Weaver, Van Duyn & Sontheimer 2005, Abdullaev et al. 2006). *In vivo*, pre-application of DCPIB has been shown to reduce neonatal brain injury and improve behavior outcomes within 24 hours of treatments in mouse pups following hypoxic induced conditions (Alibrahim et al. 2013). Despite the anion channel specificity for VRAC, DCPIB may have other effects on other ion channels such as activation of TREK potassium conductance in cultured astrocytes (Minieri et al. 2013).

While VRAC has been characterized structurally, electrophysiologically, and pharmacologically, the channel activation, pore formation, and channel gating are still not understood. Many pathways have been suggested to activate and/or enhance the current associated with VRAC during RVD, as discussed below.

C. ATP

Adenosine 5'-triphosphate (ATP) is widely known as a high energy intermediate in metabolism but is also an important extracellular and intracellular signaling molecule in the CNS. Osmotic cell swelling has been shown to release ATP from many cell types (van der Wijk et al. 2003, Espelt et al. 2013) and in cultured astrocytes, ATP released during hypoosmotic swelling acts on purinergic P2 receptors to stimulate $I_{Cl,swell}$ (Darby

et al. 2003) and excitatory amino acid release (Mongin & Kimelberg 2002). ATP can be released through conductive channels including connexin hemi-channels and pannexin channels or by exocytotic vesicular transport (Islam et al. 2012, Lohman & Isakson 2014). While the connexin- and pannexin-related release of ATP is still not entirely understood, these channel-mediated pathways of ATP release are typically driven by a steep electrochemical gradient (Espelt et al. 2013). The released extracellular ATP concentration adjacent to the plasma membrane can reach or exceed 10 μM and at this concentration ATP can stimulate purinergic signaling via P2Y receptors on the cell surface (Dubyak & el-Moatassim 1993). In primary rat astrocytes, application of hypoosmotic medium together with exogenous extracellular ATP resulted in a synergistic increase of excitatory amino acid release suggesting ATP regulates VRAC (Mongin & Kimelberg 2002). With this information, I plan to investigate the role purinergic signaling plays in VRAC activation.

P2Y and P2X purinergic receptors

Purinergic signaling has been implicated in many physiological and pathological conditions and processes including regulation of blood pressure and respiratory control, neurotransmission, inflammation, and cancer (Lohman & Isakson 2014). Purinergic receptors consist of three main classes: metabotropic P1 adenosine receptors (ARs), ionotropic P2X receptors, and metabotropic P2Y receptors (Lohman & Isakson 2014, Singh, Boyer, Der & Zohn 2010). Purinergic receptors are broadly distributed in neurons and glial cells, and these receptors have a high binding affinity to ATP and ADP nucleotides.

Seven subtypes of the ionotropic P2X receptor family and eight metabotropic P2Y receptors (P2Y1, P2Y2, P2Y4, P2Y6, P2Y11, P2Y12, P2Y13, and P2Y14) occur. Three P2Y receptors are preferentially activated by ADP: P2Y1, P2Y12 and P2Y13 (Espelt et al. 2013). P2Y2 is preferentially activated by UTP or ATP (Solfoff, Avraham, Avraham & Cantley 1998). Overall, the metabotropic P2Y receptors have been shown to couple to G_q, G_s, or G_i and modulate intracellular inositol triphosphate (IP_3), calcium, and cyclic AMP (cAMP) levels (Lohman & Isakson 2014). Recent studies which examined metabotropic P2Y receptor roles during osmotic challenge in retinal glia (Müller) cells found activation of the purinergic receptor signaling cascade mediated swelling in this cell type (Vogler et al. 2016).

ATP role in RVD

ATP stimulation of purinergic signaling through the P2Y2 receptors (P2Y2R) may play a role in modulating RVD response during cell swelling (Dezaki, Tsumura, Maeno & Okada 2000). P2Y2 receptors have been shown to be ubiquitously expressed on the plasma membrane in rodent fibroblasts (Homolya et al. 1999), in prostate cancer cells (Li et al. 2013) and cortical astroglia (Fischer et al. 2009) during homeostatic and osmotically challenged conditions (Lohman & Isakson 2014, Singh, Boyer, Der & Zohn 2010). And for astrocytes, specifically, P2Y receptors are expressed on the cell plasma membrane *in vivo* and *in vitro* (Franke et al. 2001, Zhu & Kimelberg 2001). In hepatoma cells, hypoosmotic swelling induced the release of endogenous ATP followed by the activation of P2Y receptors and then an increase in volume-dependent current (Wang, Mehta & Rose 1995). Furthermore, in the human epithelial cell line, Intestine407, ATP

release during osmotic swelling was shown to reach a concentration high enough to stimulate P2Y purinergic receptors (Hazama et al. 1999). However, the application of extracellular ATP under normal isoosmotic conditions has not been found to stimulate volume sensitive chloride currents (Nilius, Sehrer & Droogmans 1994, Jackson & Strange 1995, van der Wijk, De Jonge & Tilly 1999) suggesting purinergic activation of $I_{Cl,swell}$ requires osmotic swelling. In astrocyte cell cultures, exogenous ATP release during hypoosmotic swelling activates $I_{Cl,swell}$ through purinergic P2Y receptors (Darby et al. 2003). The swelling-induced current was maximally activated at 1 mM extracellular ATP concentration which is well above normal physiological conditions. Thus, extracellular ATP's effect on RVD is mediated, at least in part, by purinergic receptor activation (Espelt et al. 2013).

ATP release pathways

Because purinergic receptors are activated by extracellular nucleotides, drugs which cause hydrolysis of extracellular ATP, inhibit the purinergic receptor, or limit ATP release may be tools for studying purinergic receptor activation during osmotic cell swelling. Extracellular ATP and ADP concentrations can be reduced by various drugs including apyrase, suramin, meclofenamate, and carbenoxolone. When added to the intracellular or extracellular solutions in patch clamp studies, these drugs work by either hydrolyzing ATP and ADP nucleotides to adenosine monophosphate (AMP) and inorganic phosphate, competitive inhibition of purinergic receptors, or blocking nucleotide release. Studies using Intestine407 cells have found extracellular application of ATP enhanced RVD whereas the application of apyrase, an ATP hydrolyzing enzyme,

inhibited RVD (Dezaki, Tsumura, Maeno & Okada 2000). This behavior was also shown by Espelt and colleagues in which they demonstrated that apyrase inhibits the swelling-induced chloride current while ATP and P2Y agonists stimulate this same current in Huh-7 hepatoma cells (Espelt et al. 2013).

Suramin, a G-protein antagonist, was also shown to suppress RVD during cellular swelling (Galietta et al. 1997). Suramin inhibits the Gα and Gβγ interface of the GPCR in rat lung cell membranes (Chung & Kermode 2005). Suramin has also been shown to inhibit volume-dependent taurine release in hippocampal neurons (Li & Olson 2004). Suramin and its analogues are direct antagonists of heterotrimeric G-proteins and have been found to act as quasi-competitive stimuli; that is, increasing the agonist concentration overcomes suramin inhibition (Freissmuth, Waldhoer, Bofill-Cardona & Nanoff 1999, Beindl et al. 1996). However, in human Intestine407 cells, suramin treatment also interacts with the channel protein responsible for volume sensitive chloride current unrelated to the drug's role as a purinoreceptor blocker (Galietta et al. 1997; van der Wijk, De Jonge & Tilly 1999). van der Wijk and colleagues' research performed on human Intestine407 cells treated with suramin and reactive blue suggested the channel responsible for $I_{Cl,swell}$ may share a homologous nucleotide-binding structure with purinergic receptor proteins (van der Wijk, De Jonge & Tilly 1999). Similar observations of the effects of extracellular ATP, suramin, and apyrase on RVD also have been found in other cell types (Leal Denis et al. 2016, Dezaki, Tsumura, Maeno & Okada 2000, Wang, Roman, Lidofsky & Fitz 1996).

Hemi-channels consisting of the gap junction proteins, connexins, have been suggested as major ATP release channels (Locovei, Bao & Dahl 2006, Blum, Walsh & Dubyak 2009,

Thompson, Zhou & MacVicar 2006, Lohman & Isakson 2014). Connexins were first described in 1970 as the principle protein making up a hemi-channel (Goodenough & Revel 1970). Connexin monomers hexamerize to form half of a gap junction channel or a hemi-channel on the plasma membrane. The hexamerized proteins dock with adjacent cell hemi-channels to form a gap junction (Lohman & Isakson 2014). While gap junctions act as a direct conduit between cells transferring intracellular ions and small molecules such as ATP and aid electrical coupling between progenitor cells, neurons, and glial cells, it has been proposed that certain isoforms of undocked connexin hemi-channels may provide an efflux route for intracellular ATP (Lazarowski 2012).

Another family of channel forming proteins, pannexins, have been shown to oligomerize and form similar hexameric channels to those formed by connexins. However, these proteins do not form gap junction channels with adjacent cells and instead form single membrane channels. Because of the structure similarity to hemi-channel proteins, pannexin channels have also been implicated in extracellular ATP release (Sosinsky et al. 2011).

Several drugs have been shown to inhibit flux of ions and osmolytes through connexin and pannexin hemi-channels (Tamura et al. 2011). Carbenoxolone is a well known blocker of conductive ATP release through these gap junction protein hemi-channels. Application of carbenoxolone during O_2/Glucose deprivation studies using acutely isolated hippocampal neuron preparations found carbenoxolone blocks hemi-channel conductance in neurons (Thompson, Zhou & MacVicar 2006).

Meclofenamate also suppresses ATP release via hemi-channels. Meclofenamate, belonging to the fenamate class, are widely used as a non-steroidal anti-inflammatory drug. Fenamates serve as cyclooxygenase inhibitors and also have been shown to play an inhibitory role in ion channel activation including voltage gated potassium and calcium channels (Li, Vapaatalo, Vaali & Paakkari 1998), ATP-sensitive potassium channels (Grover et al. 1994), and gap junctions (Harks et al. 2001). Along with its role as a connexin hemi-channel inhibitor, meclofenamate also has been suggested as a blocker of ATP sensitive potassium channels (K_{ATP}) in cardiac cells (Grover et al. 1994). Patch clamp studies using SKHep1 cells overexpressing connexin43 (Cx43) revealed that meclofenamate also inhibited the Cx43-mediated intracellular communication (Harks et al. 2001).

D. G-proteins and G-protein Coupled Receptors

Intracellular signaling mechanisms involve a molecule or stimulus which acts upon a receptor molecule capable of perceiving the signal. This activation then leads to a second messenger with further signaling cascades involved in normal cell processes. Many of these signaling cascades in mammalian cells involve G-proteins, G-protein coupled receptors (GPCRs), and effectors. GPCRs comprise the largest group of cell surface receptors with over 1000 members, thus representing approximately 1% of the total proteins encoded by the human genome (Schulte & Levy 2007). The common structural model for the receptors consists of seven transmembrane regions (Lodish et al. 2000, Tuteja 2009). GPCRs are activated by a variety of stimuli including growth factors, neurotransmitters, hormones and chemoattractants. Activation of the receptor leads to a

conformational change in the transmembrane α helices, and this conformational change causes an exchange of GDP for GTP bound to the G-Protein α subunit. G-proteins are transmembrane proteins coupled to cell surface receptors that act as secondary messengers within the cell.

G-proteins consist of two main classes: heterotrimeric and GTPases. Heterotrimeric G-proteins consist of 3 subunits: alpha, beta, and gamma. This trimer is inactive when reversibly bound to GDP. To mediate an intracellular event, the protein binds to GTP to form an active complex. Hydrolysis of GTP by the alpha subunit alters the beta gamma subunit and causes it to dissociate from the target protein. G-proteins then cycle between the active GTP-bound state and the inactive GDP-bound state. Further signaling transduction depends on the type of G-protein involved. Because trimeric G-proteins are versatile, different receptor-hormone complexes can modulate activity for the same effector protein.

The four main alpha subunits are: s, i, q, 12/13. The alpha s subunit has been found to stimulate the adenylyl cyclase (AC) pathway while the activation of the alpha i subunit has been shown to inhibit this same pathway. Activation of the alpha q subunit leads to the stimulation of the PIP2 pathway. Lastly the alpha 12/13 subunit acts on the Rho GTPase pathway. Rho-GTPase signaling pathways, independent of phosphorylation events, have also been shown to regulate swelling induced chloride current in bovine endothelial cells (Nilius et al. 1999).

G-protein subunits display an array of molecular diversity which has not been fully understood (Beindl et al. 1996). In more recent years, GPCRs have been found to

modulate potassium, calcium, and sodium voltage-gated ion channel activity in neurons and other excitable cells (Abbracchio et al. 2006). And more recently, GPCR-regulated ATP release has been shown to increase during hypoosmotic swelling in 1321N1 astrocytoma cells (Blum, Walsh & Dubyak 2009). Previous studies using native 1321N1 astrocytoma cells (which do not express functional purinergic receptors) found swelling does not activate chloride currents and RVD does not occur following osmotic swelling (Wenker & Olson 2008). Thus, a G-protein pathway initiated by purinergic signaling may play a role in the signaling cascade involved with VRAC activation.

GTP is a purine nucleoside triphosphate well known for its involvement in DNA synthesis but also through its role in G-protein activation and signal transduction. In various cell types, application of GTPγS, a non-hydrolyzable GTP analog, has been shown to increase chloride conductance (Doroshenko 1991, Tilly et al. 1991, Nilius et al. 1999), and this application of GTPγS has been shown to activate an outwardly rectifying current independent of swelling (Doroshenko & Neher 1992, Estevez, Bond & Strange 2001). In calf pulmonary artery endothelial cells, VRAC is activated by GTPγS under isoosmotic conditions (Voets et al. 1998). And in cervical cancer cells, extracellular GTPγS application was found to facilitate the RVD process. Estevez and colleagues found that $I_{Cl,swell}$ stimulation did not require phosphorylation mechanisms and was inhibited by GDPβS (another non-hydrolyzable GDP analog that competes with the GTP binding site on the G-proteins) and *Clostridium difficile* toxin B (Estevez, Bond & Strange 2001). *Clostridium difficile* toxin B (TcdB) is a potent cytotoxin which interacts with small GTP binding proteins: Rho, Rac and Cdc42 (Voth & Ballard 2005).

Another toxin which affects GPCR responses is pertussis toxin. Pertussis toxin (PTX) is an A-B exotoxin that inhibits G_0 and G_i proteins via ADP-ribosylation. PTX acts by preventing G-proteins from interacting with their GPCR by locking the α subunit in an inactive state (Mangmool & Kurose 2011). Research has shown PTX may play a therapeutic role in hypertension in rats (Kost, Herzer & Jackson 1999) and has been shown to inhibit inwardly rectifying potassium channels in HEK293 cells (Leaney & Tinker 2000). Researchers have also found that volume-activated taurine efflux mediated by an anion channel in human cervical cancer cells HT-3 was inhibited by pertussis toxin application (Chou, Shen, Chen & Huana 1997) suggesting pertussis toxin may be blocking the VRAC channels involved in RVD. Thus, G-protein coupled receptors and G-proteins may be implicated in the signaling cascade involved with VRAC activation during cell swelling.

GPCR control of VRAC involves an increase in intracellular calcium ions, but it is unclear whether intracellular calcium release is involved in RVD and whether these changes involve purinergic signaling (McCarty & O'Neil 1992, Roe, Moore & Lidofsky 2001). However, this increased intracellular Ca^{2+} may be involved in PKC activation through Ca^{2+} signaling cascades (Rudkouskaya, Chernoguz, Haskew-Layton & Mongin 2008). Protein kinase C (PKC) is a family of kinase enzymes that control protein function by phosphorylation of the hydroxyl groups on serine and threonine amino acid residues. PKC is widely expressed in all eukaryotic cells and has been shown to be involved in EAA release (Rudkouskaya et al. 2008). However, there is controversy over the role of PKC during ischemic injury. Some studies have shown up-regulated PKC-dependent neuroprotective pathways while others have found a loss in PKC activity following acute

ischemic injury. These conflicting reports may be tied to varying animal models used, intensity of the ischemic onset, and the different PKC isozymes studied (Bright & Mochly-Rosen 2005). PKC may play a role in VRAC activation and RVD response following ischemic injury, but further studies are needed to determine this role.

E. Protease Activated Receptors

Another G-protein coupled receptor (GPCR) implicated in controlling cellular growth, neuronal death, proliferation and inflammation, is the protease activated receptor (PAR) family of proteins (Striggow et al. 2000). PARs are irreversibly activated by proteolytic cleavage of the extracellular *N* terminus. The remaining amino terminus then functions as a tethered ligand binding to the receptor which then irreversibly activates the PAR signal transduction pathways. The PAR family of receptors consists of four known members: PAR1, PAR2, PAR3, and PAR4. PAR1 was the first receptor discovered through its interaction with thrombin. PAR1, PAR3, and PAR4 are activated by the coagulant protease, thrombin, while PAR2 is activated by trypsin-like serine proteases such as trypsin, tryptase, and upstream coagulation proteases, factors VIIa and Xa. PAR2 is not activated by thrombin.

PAR downstream effects (G-protein pathways)

The serine protease, thrombin, is well known for its role in blood coagulation, however, thrombin has also been implicated in synaptic plasticity and neurodegenerative disorders such as Alzheimer's dementia. Thrombin is generated from prothrombin during cell

injury through either an extrinsic or intrinsic signaling cascade involving various blood clotting proteins: tissue factor (TF) X, XI, XII, III, and V (Monroe, Hoffman & Roberts 2002). During acute brain injury, cells in the CNS are exposed to this serine protease. Thrombin works by activating a phospholipase C (PLC) signaling cascade. In the majority of cell types, PAR1 and PAR2 are often co-expressed on the cell membrane and are involved in regulating downstream signaling pathways (Ubl, Vohringer & Reiser 1998, McCoy, Traynelis & Hepler 2010). Activation of the PAR1 receptor is capable of activating G-protein families: G_i, G_1 and $G_{12/14}$ (Sorenson et al. 2003). Further research has found that PAR1 activation from these G-protein pathways is involved in mediating cell proliferation in rat primary astrocytes (Wang et al. 2002).

PAR actions on RVD and other functions

Studies using mouse brain slices have shown an upregulation of PAR1 during ischemic conditions (Wang et al. 2012). It has been shown that thrombin has a neuroprotective effect during cerebral ischemia when injected at a low dose, but at higher doses, thrombin application has been shown to cause cell apoptosis and act more as a neurotoxin (Striggow et al. 2000, Donovan & Cunningham 1998, Xi, Reiser & Keep 2003). PAR1 upregulation during acute ischemia also has been associated with apoptosis in cultured neurons and astrocytes and with glial cell proliferation. This thrombin-induced neuronal cell death was associated with activation of RhoA. Activation of RhoA was also found to be inhibited by tyrosine kinase inhibitors suggesting tyrosine kinase is required upstream in the signaling cascade (Donovan, Pike, Cotman & Cunningham 1997, Donovan & Cunningham 1998).

PAR has been found to increase ATP release during cell swelling. Thrombin activation of PAR receptors in 1321N astrocytoma cells causes ATP release under isoosmotic conditions and this release is enhanced during hypoosmotic stress (Joseph, Buchakjian & Dubyak 2003). Blum and colleagues found that the osmotically enhanced PAR1-dependent ATP release in the 1321N1 cell type was completely eliminated under hypertonic conditions (Blum, Walsh & Dubyak 2009). This suggests that ATP release following PAR receptor activation is osmotically dependent. PAR1 receptors also have been shown to reduce the threshold osmolarity for taurine release in 1321N1 astrocytoma cells (Cheema, Ward & Fischer 2005).

F. Summary of Background Literature

Volume regulated anion channels (VRAC) play an important role in regulatory volume decrease (RVD) following hypoosmotic stress. VRAC has been implicated as the channel responsible for $I_{Cl,swell}$ (Voss et al. 2014). Because chloride is the most abundant anion in the cell, this channel is a major contributor to volume regulation. While the channel is characterized electrophysiologically, pharmacologically, and more recently, structurally, not much is known regarding channel activation, pore formation, membrane recruitment, and the upstream regulators involved. In this study, I am specifically concerned with VRAC activation in brain cells and the possible upstream regulators of VRAC gating and activation such as G-proteins, ATP, and the protease activated receptors.

Extracellular ATP has been suggested to regulate VRAC during hypoosmotic exposure in primary rat astrocytes (Mongin & Kimelberg 2002). Other research has shown a

thrombin-enhanced ATP release in 1321N1 cells *in vitro* that is sensitive to osmotic conditions (Blum, Walsh & Dubyak 2009); however, extracellular ATP has not been found to stimulate VRAC under isotonic conditions (Nilius, Sehrer & Droogmans 1994, Jackson & Strange 1995). Thus, I will investigate the roles of GPCR signaling, purinergic signaling through P2Y2 receptors, PAR1 activation, and extracellular ATP-dependent pathways on $I_{Cl,swell}$ during RVD.

As shown in Figure 1, I propose that PAR1 activation via thrombin and/or P2Y2 receptor activation by exogenous ATP will activate VRAC. I propose that inhibition of these pathways will inhibit swelling-induced chloride currents through this channel in this tumor cell type.

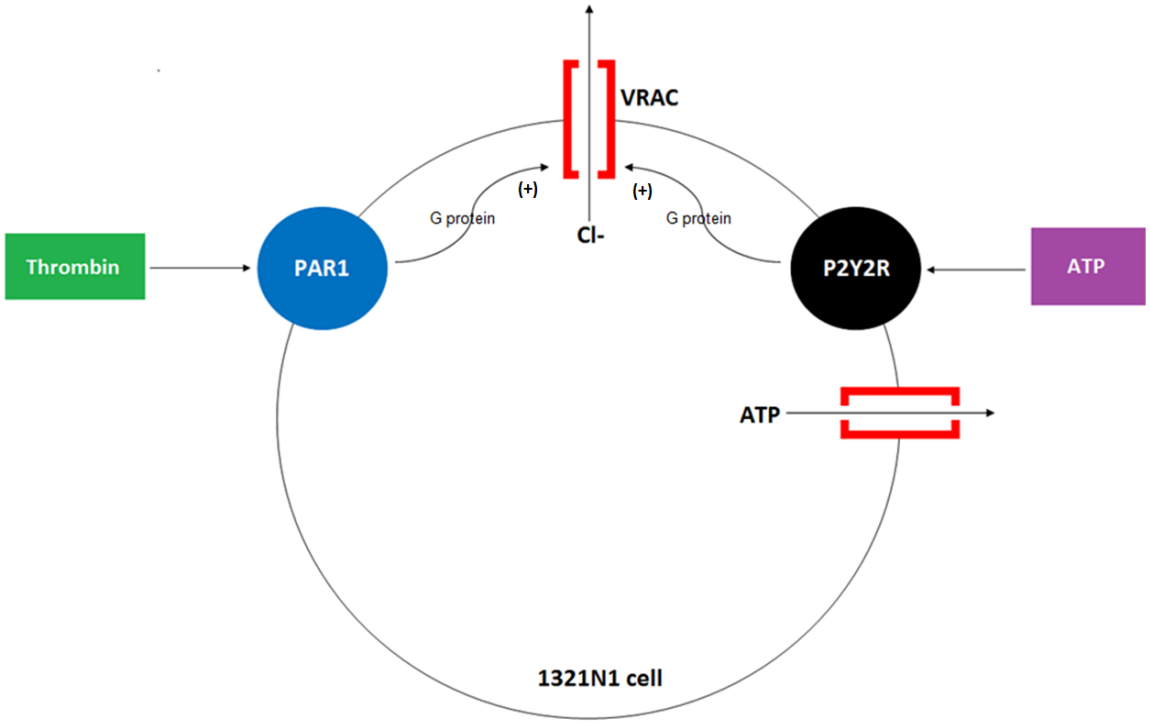


Figure 1: Schematic view of proposed upstream regulators of VRAC during cell swelling

II. Specific Aims

Specific Aim I

Identify and characterize VRAC in a P2Y2R-transfected astrocytoma cell line. VRAC will be pharmacologically and electrophysiologically identified.

Specific Aim II

Determine if VRAC can be activated under isoosmotic conditions using ATP and other G-protein coupled receptor activators.

Specific Aim III

Determine if VRAC activation during hypoosmotic exposure is G-protein coupled. This will be addressed pharmacologically using exogenous ATP and thrombin application, G-protein activators and G-protein inhibitors and through the addition of pertussis toxin intracellularly.

III. Materials and Methods

A. Abbreviations

ADP = adenosine 5'-diphosphate, **ATP** = adenosine 5'-triphosphate, **ClC-*X*** = chloride channel family, **CNS** = central nervous system, **CsCl** = cesium chloride, **DCPIB** = 4-[(2-Butyl-6,7-dichloro-2-cyclopentyl-2,3-dihydro-1-ox o1H-inden-5-yl)oxy]butanoic acid, **DIDS =** 4,4'-Diisothiocyanatostilbene-2,2'-disulfonic acid, **DMEM** = Dulbecco's modified Eagles medium, **DMSO** = dimethyl sulfoxide, **EGTA** = ethylene glycol tetraacetic acid, **FBS** = fetal bovine serum, **GDP** = guanosine 5'-diphosphate, **GPCR** = G-protein couple receptor, **GTP** = guanosine 5'-triphosphate, **HEPES** = 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, **NKCC** = $Na^+/K^+/2Cl^-$ symporter, **NPPB** = 5-nitro-2-(3-phenylpropyl-amino) benzoic acid, **PAR** = protease activated receptor, **PBS** = phosphate buffer solution, **PKC** = protein kinase C, **PTX** = pertussis toxin, **RVD** = regulatory volume decrease, **RVI** = regulatory volume increase, **SITS =** 4-Acetamido-4'-isothiocyanatostilbene-2,2'-disulphonic acid, **VRAC** = volume regulated anion channel.

B. Materials

The following materials were used to perform the experiments in this thesis: low-glucose DMEM, thrombin from bovine plasma, geneticin (G418), DMSO, HEPES, EGTA, Na_2-

ATP, pertussis toxin lyophilized powder, meclofenamate sodium, carbenoxolone disodium salt, and suramin sodium salt were purchased from Sigma-Aldrich (St. Louis, MO), Penicillin/streptomycin, trypsin 0.25%, and FBS were purchased from Invitrogen (Carlsbad, CA), and DCPIB purchased from Tocris Biosciences (Ellisville, MO)

Other chemicals used were CsCl and CsOH purchased from Sigma-Aldrich (St. Louis, MO), $CaCl_2$ and dextrose purchased from J.T. Baker Chemical Co. (Phillipsburg, NJ), and $MgCl_2$ purchased from MCB Reagents (Cincinnati, OH).

C. Cell Culture

The cells used in the experiments are transgenic clones of 1321N1 human astrocytoma cells which express the human P2Y2 receptor. These cells had been stably transfected with the human P2Y2 receptor subtype (P2Y2 cells) and simultaneously transfected with resistance to geneticin (G418), an aminoglycoside antibiotic similar in structure to gentamicin that blocks ribosomal protein synthesis in prokaryotic and eukaryotic cell lines. The cells were a gift by Natalia Chorna, Ph.D. (University of Puerto Rico, San Juan, Puerto Rico) and were shipped in low-glucose Dulbecco's modified Eagles medium (DMEM). They were replated upon arrival in growth media containing low-glucose DMEM, 5% FBS, 1% penicillin/streptomycin, and 0.5 g/L G418. At 90% confluency, cells were removed and resuspended in similar growth media but containing 10% DMSO and 20% FBS before being cryogenically frozen at 77ºK in 1 mL aliquots for stable long-term storage.

After rapidly thawing, cells were cultured in plastic petri dishes with growth media and incubated in a humidified atmosphere containing 5% CO_2 and 95% air at 37ºC. Growth media was replaced every 3-4 days and once the cells reached 90% confluency, they were replated. Replating of cells consists of treating the cells with 0.25% trypsin solution for 5-10 minutes to loosen the cells from the surface. The resulting cell suspension then was centrifuged for 5 minutes at 300 × g. Then the trypsin solution was removed through aspiration and the remaining pellet resuspended in fresh growth medium. A fraction of the suspension was then replated with additional growth media. Cells used for patch clamp experiments were plated two days prior to the electrophysiological recordings on sterilized 12 mm coverslips placed in the petri dishes.

D. Electrophysiological Recording

Coverslips with attached cells were placed on the stage of a Nikon TMS-F inverted phase contrast microscope (Tokyo, Japan) containing a single-coverslip chamber (Warner Dual Heater Controller TC-344, Hamden, CT). Coverslips were perfused with a phosphate buffer solution (PBS) using a Cell-Micro Controls perfusion system (Norfolk, VA) at a rate of 2-3 mL/min. The PBS solution contained (in mM): NaCl (147), KH_2PO_4 (0.5), Na_2HPO_4 (3.2), $CaCl_2$ (1.0), $MgCl_2$ (0.5), dextrose (5.0), KCl (2.7) and pH was adjusted to 7.3 using NaOH. The final osmolarity of the solution was adjusted to 290 mOsm using small volumes of 3 M NaCl and confirmed with a Wescor vapor pressure osmometer (Logan, UT). The chamber temperature was measured with a thermocouple and held at 35ºC using integral heating elements. Excess fluid from the perfusion system was removed via a vacuum system to maintain a constant fluid level.

Patch pipettes were pulled in two stages from thin-walled glass capillary tubes (World Precision Instruments Inc., Sarasota, FL) using a Narishige PP-83 puller (East Meadow, NY). The electrode was filled with a CsCl solution containing (in mM): CsCl (100), $CaCl_2$ (1.0), $MgCl_2$ (1.0), EGTA (10), HEPES (10), Na_2ATP (5.0), glucose (5.5), and approximately 100 mM of sucrose with a final osmolarity adjusted to 290 mOsm using sucrose and pH was adjusted to 7.3 using CsOH. When filled with a CsCl solution, patch pipettes had a resistance of 3-5 MΩ.

Whole cell recordings were initiated while PBS perfused the recording chamber. The electrode was lowered into the bath solution and manipulated so that the tip was adjacent to the cell membrane. While the pipette tip was in this solution, the pipette offset was set to 0 mV and the pipette resistance measured at a holding membrane potential of 0 mV using an Axopatch 200A amplifier (Union City, CA) controlled with Clampex 2.0 software (Union City, CA). The electrode was then advanced toward the cell surface. Upon contact with the cell, light suction was applied to the electrode solution to draw the cell membrane into the pipette tip. Once the pipette resistance was greater than one gigaohm, more suction was applied to break the cell membrane that had been drawn into the pipette. In all experiments, cell capacitance then was measured in Clampex 8.2 software (Union City, CA) while holding the cell in PBS solution. Capacitance was used to normalize the measured whole cell currents to current density units of nA/pF.

Within two minutes of obtaining a stable whole cell recording, the bath perfusion solution was switched to isoosmotic CsCl solution consisting of (in mM): CsCl (100), $CaCl_2$ (1), $MgCl_2$ (0.5), HEPES (10), sucrose (90), glucose (5.5). The membrane potential for all experiments was held in voltage clamp mode at 0 mV using an Axopatch 200A Axon

Instruments amplifier (Union City, CA). A series of 100 msec voltage command pulses was applied to the cell membrane every 30 sec in a stepwise fashion from -100 mV to +120 mV or from -90 mV and +130 mV in increments of 20 mV. After 3-5 min of perfusion in isoosmotic CsCl solution, the perfusion solution was changed to a hypoosmotic CsCl solution with an osmolality of either 250 mOsm or 200 mOsm. These hypoosmotic solutions were identical to isoosmotic CsCl solutions except that the concentration of sucrose was lowered to achieve the desired solution osmolality.

In some experiments, DCPIB, ATP, thrombin, carbenoxolone, meclofenamate, or suramin were added to the CsCl perfusion solution. And in other experiments, pertussis toxin was added to the CsCl pipette solution

E. Data Analysis and Calculations

Whole cell currents were recorded using Axon Instruments Clampex 2.0 software (Union City, CA). Current measurements collected between 5 msec and 8 msec after the start of each voltage step were averaged using Axon Instruments Clampfit 8.2 software (Union City, CA) and then transferred to Microsoft Excel for analysis. These data points were chosen to obtain the maximum current prior to inactivation of VRAC which appears after the first 10 msec of the voltage pulse. Data for cells in isoosmotic CsCl solution were taken approximately 3 minutes after the start of the perfusion in this solution and data for the hypoosmotic treatments was taken at 5-6 minutes after the solution was changed. Cell current density was expressed in units of nA/pF determined by dividing the membrane

current (I) by the total cell capacitance (C). Current density voltage plots were constructed for the data and standard error bars were calculated from the data sets.

F. Data Analysis

Current density measurements measured at +120 mV or +130 mV were used for analysis. Data were analyzed by ANOVA or Students t-test for paired or unpaired samples with significance indicated for $p<0.05$ for a two-tailed distribution. All values are reported as the mean ± SEM. Clampfit 8.2 (Axon Instruments, Union City, CA) software was used for this statistical analysis.

IV. Results

A. P2Y2 transfected astrocytoma cells exhibit swelling-induced chloride conductance

The electrophysiological response to hypoosmotic exposure was evaluated by the voltage clamp technique. Under whole-cell voltage clamp conditions with the CsCl pipette solution and the cells perfused with isoosmotic (290 mOsm) PBS solution, cell capacitance was measured. Capacitance is a good approximation tool for biological membranes (Alvarez & Latorre 1978) and is used to normalize cell membrane surface area/size. The astrocytoma cells displayed capacitances between 20 and 80 pF.

Currents observed during isoosmotic (290 mOsm CsCl) perfusion were small in magnitude, slightly outwardly rectifying, and displayed little to no time dependent inactivation (Figure 2). The cells demonstrated an increase in current amplitude within 3-5 minutes of hypoosmotic exposure. This hypoosmotically activated current showed time inactivation at depolarized potentials. The swelling-induced current was also found to be present when the cell was perfused with 250 mOsm CsCl as shown in Figure 2. The current activated during the 250 mOsm CsCl perfusion showed slightly less maximum activation at positive potentials suggesting the magnitude of the current is related to the degree of cellular swelling.

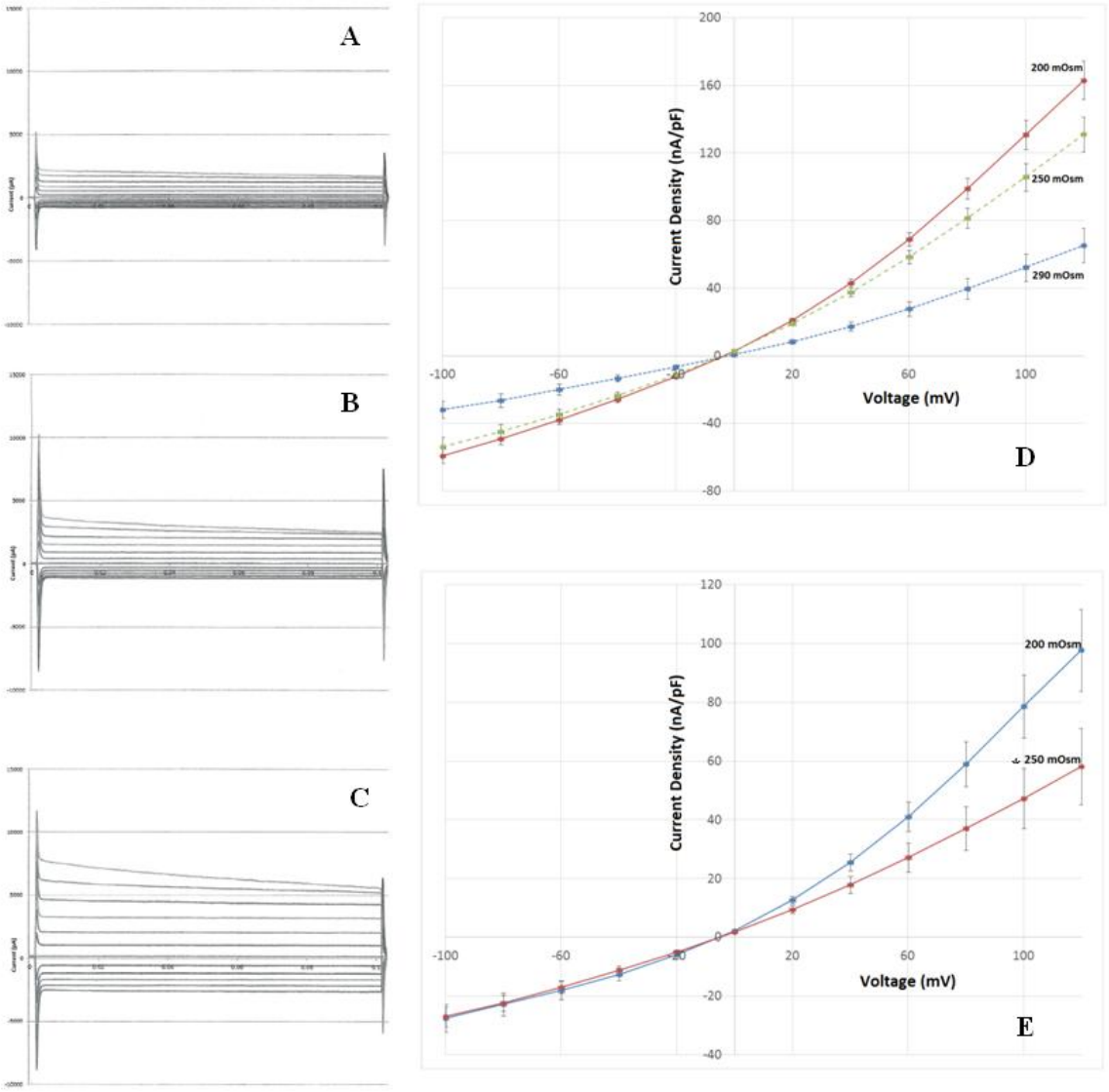


Figure 2: VRAC is present in 1321N1 astrocytoma cells transfected with P2Y2 receptors. A) Cell currents recorded while the cell was perfused with 290 mOsm CsCl solution, B) perfused with 250 mOsm CsCl solution, and C) perfused with 200 mOsm CsCl. Current density was calculated using the capacitance of each cell and an average of the cell recordings was used to create the I-V chart. D) Current-voltage relationships for isoosmotic 290 mOsm exposure, hypoosmotic 200 mOsm exposure and hypoosmotic 250 mOsm exposure. E) Swelling-activated current for 200 mOsm CsCl exposure (n=11) and 250 mOsm CsCl exposure (n=4). The (*) indicates swelling-activated current in 200 mOsm is statistically larger than the current in 250 mOsm by two-way ANOVA.

B. The current activated by hypoosmotic exposure is DCPIB-sensitive

To determine if this swelling-activated current is associated with VRAC, DCPIB (100 μM) was added to the bath solution for 2-3 minutes prior to changing from isoosmotic to hypoosmotic (200 mOsm) CsCl solution and was present throughout the isoosmotic and hypoosmotic exposure. Whole-cell recordings show the current normally activated during hypoosmotic treatment was greatly diminished when DCPIB is present in the bath solution (Figure 3) suggesting that DCPIB inhibits the channel or upstream regulators involved with channel activation.

C. VRAC is inhibited by G-protein inhibitors

To determine if G-proteins or G-protein coupled receptors are involved in VRAC signaling and/or activation, 100 μM suramin, a G-protein antagonist, was added to the bath solution during isoosmotic and hypoosmotic treatments. Figure 3 shows that adding suramin has no significant effect during 290 mOsm CsCl treatments. However, VRAC was significantly inhibited when suramin was present during perfusion with the 200 mOsm solution ($p=0.0024$).

Addition of 1 μg/mL pertussis toxin to the patch solution gave similar results to those observed with extracellular suramin treatments. Pertussis toxin had no effect during isoosmotic CsCl perfusion, but significantly reduced the VRAC current during hypoosmotic exposure.

D. Thrombin enhances chloride conductance during cell swelling

Thrombin is a serine protease found to activate three of the known G-protein coupled protease activated receptors (PAR): PAR1, PAR3, and PAR4. To determine if PAR can enhance the RVD response of VRAC, 5 U/mL thrombin was added to isoosmotic and hypoosmotic (200 mOsm) perfusion solutions (Figure 3). Thrombin added during isoosmotic perfusion did show a significant effect on membrane current ($p=0.0105$). Additionally, with thrombin present during hypoosmotic exposure, a significant increase in the magnitude of the hypoosmotic current was also observed ($p=0.0005$).

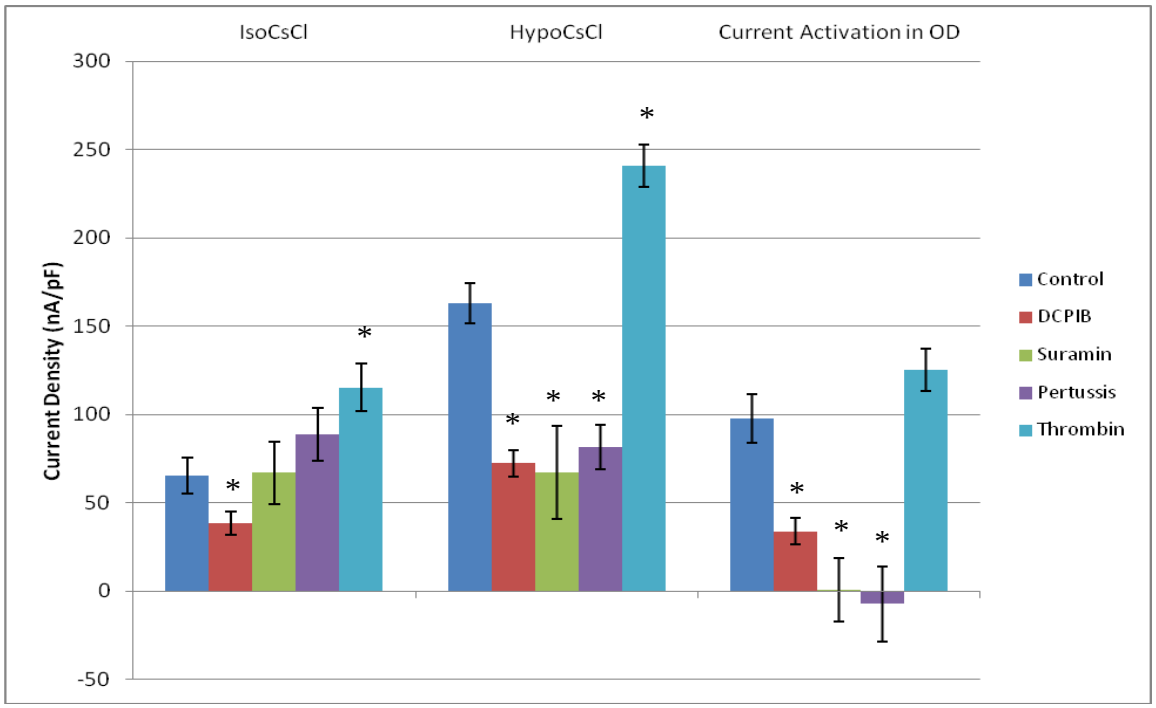


Figure 3: Drugs that act on VRAC conductance. For control recordings, current was measured while cells were perfused in isoosmotic 290 mOsm CsCl solution and hypoosmotic 200 mOsm CsCl bath solution without added drug (n=11). Current density was calculated using the capacitance of each cell and an average was taken at +120 mV holding potential. When DCPIB (100 µM, n=6), suramin (100 µM, n=5), or thrombin (5 U/mL, n=5) was used, the drug was present in both the isoosmotic and hypoosmotic perfusion solutions. Pertussis toxin (1 µg/mL, n=7) was added to the CsCl pipette solution during both isoosmotic and hypoosmotic exposure. Error bars show the standard error of the mean for each set of data. The (*) indicates significance ($\alpha<0.05$) in comparison to control recordings.

E. Endogenous ATP does not affect VRAC activation

To determine the effect of exogenously applied ATP on the swelling-activated current, 100 μM ATP was added to the isoosmotic CsCl perfusion solution and was present at the same concentration in the hypoosmotic (200 mOsm) CsCl perfusion solution. Figure 4 shows 100 μM exogenous ATP does not significantly enhance the conductance during perfusion with 290 mOsm CsCl solution. Similarly, there was no significant effect on the swelling-induced current in hypoosmotic 200 mOsm CsCl perfusion solution.

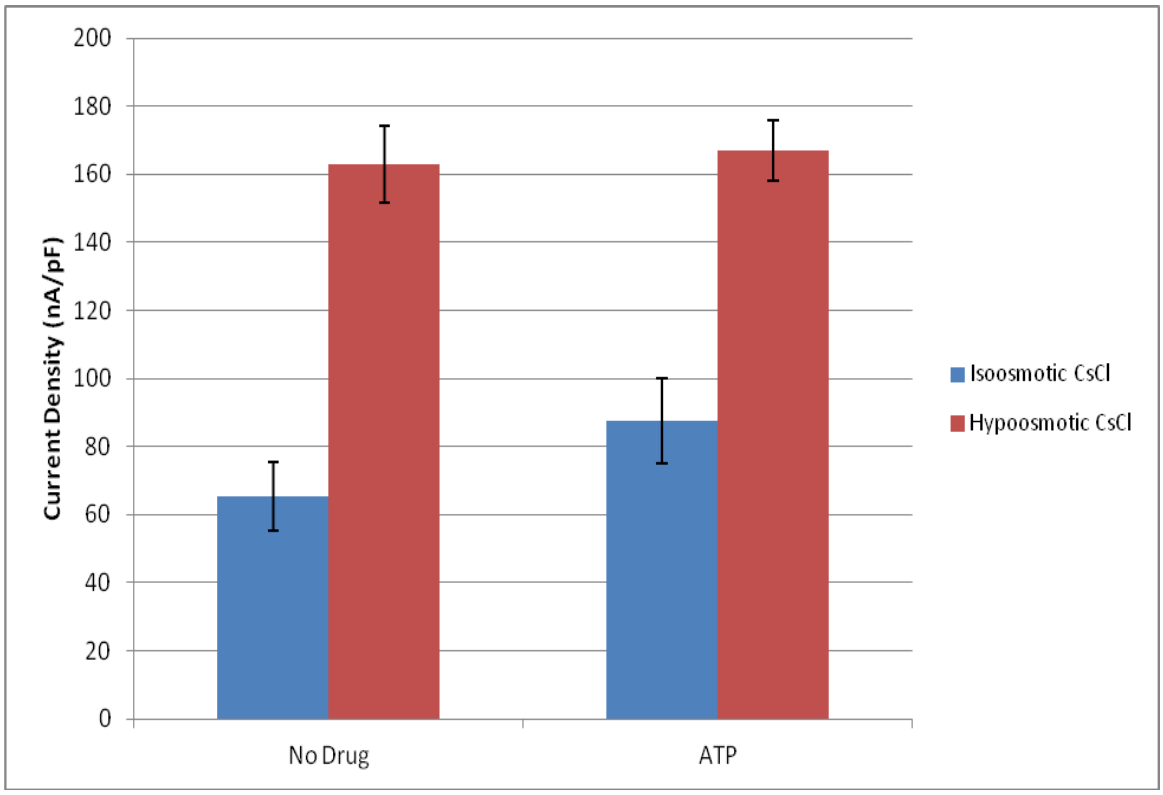


Figure 4: The effect of exogenous ATP on VRAC. For isoosmotic and hypoosmotic recordings, current was measured while cells were perfused in 290 mOsm CsCl solution and 200 mOsm CsCl perfusion solution, respectively. Current density was calculated using the capacitance measured for each cell and the average current measured at +120 mV. For control recordings, currents were measured in the absence of exogenous ATP Data from the ATP group are from cells exposed to 290 mOsm CsCl (isoosmotic) and 200 mOsm CsCl (hypoosmotic) solutions in the presence of 100 µM ATP Values are the mean for 11 cells in control and 9 cells in ATP groups. Error bars show the standard error of the mean for each set of data.

F. Limiting ATP release pathways had no effect on VRAC conductance

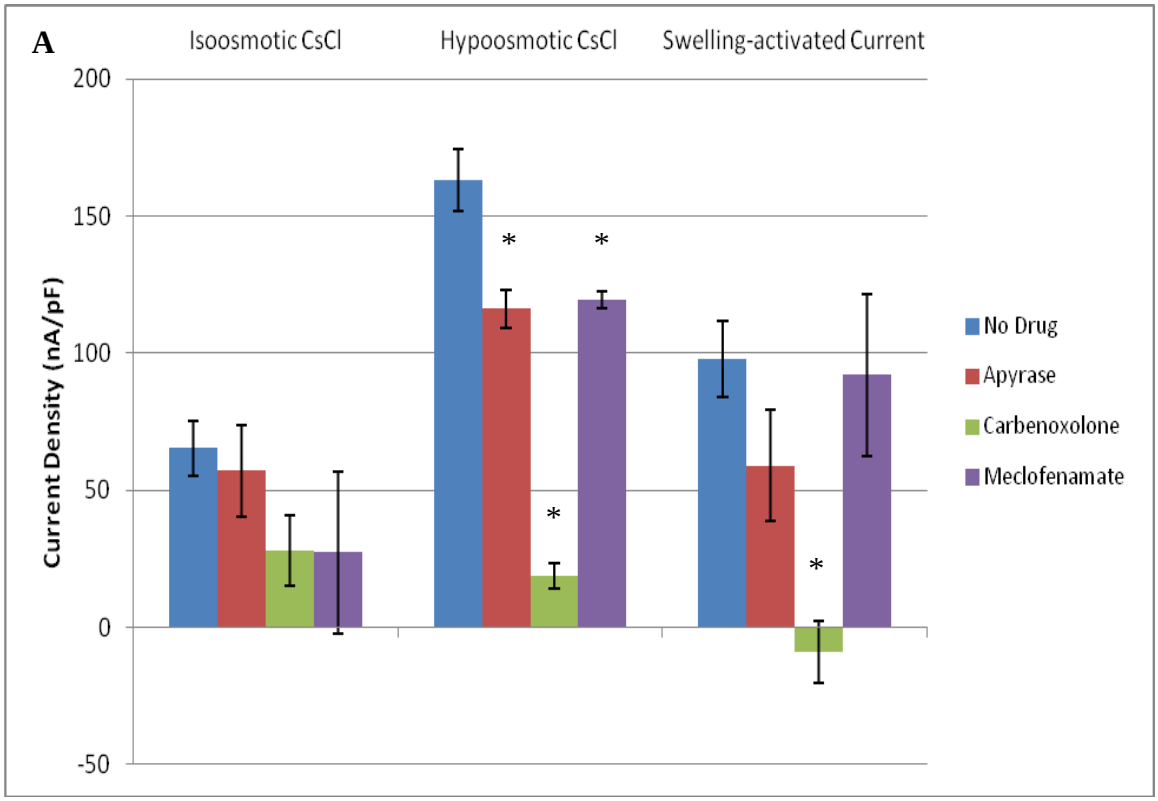
I tested if reducing the extracellular ATP concentration or limiting cellular ATP release pathways that are activated by cell swelling would affect VRAC activation.

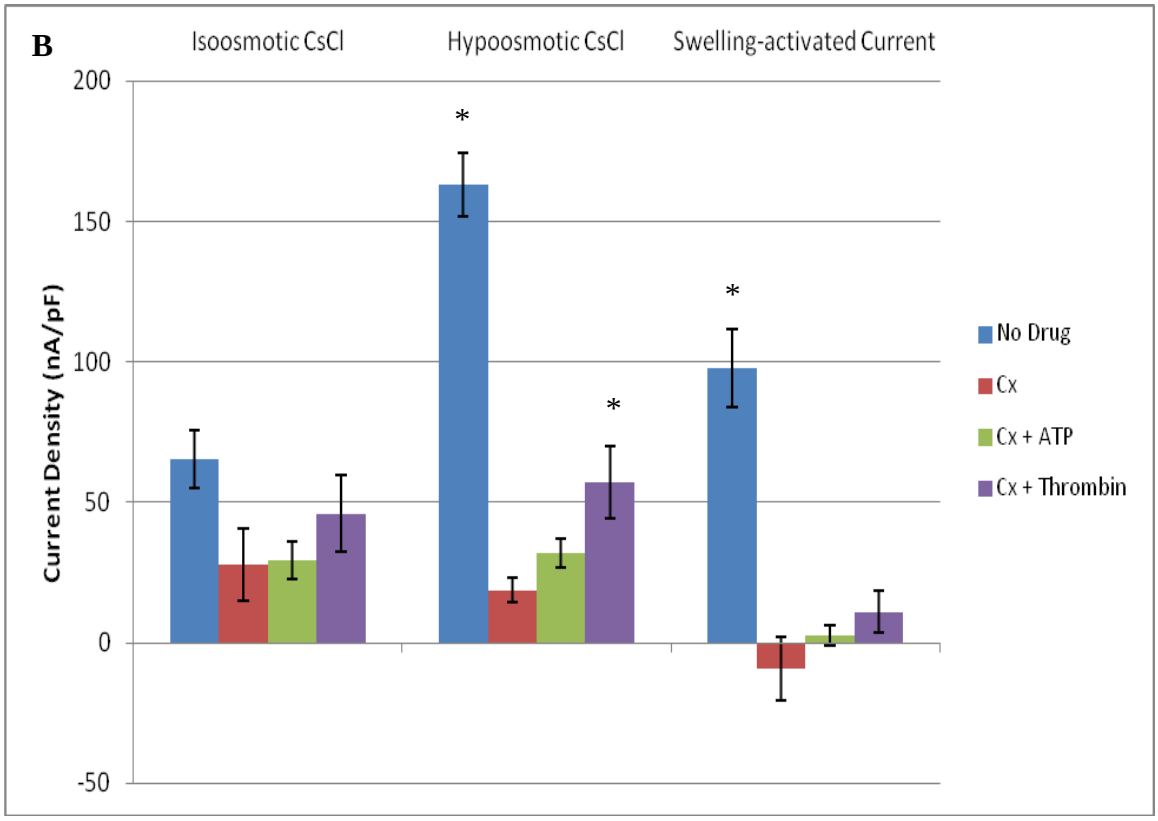
Apyrase, an ATP hydrolyzing enzyme, was added to the bath solutions and current density was measured at +120 mV. Figure 5A shows 5 U/mL apyrase added to the 290 mOsm solution had no effect on whole cell current density. However, apyrase reduced the hypoosmotic current when present in the 200 mOsm bath solutions ($p=0.0314$).

Meclofenamate and carbenoxolone inhibit conductance of gap junction hemi-channels and by doing so reduce ATP release during cell swelling (Harks et al. 2001, Thompson, Zhou & MacVicar 2006). When 100 μM meclofenamate was present during hypoosmotic exposure, there was no significant effect on the swelling-activated current. However, when 100 μM carbenoxolone was added to the 200 mOsm solution, I saw a significant inhibitory effect on this current ($p=0.0001$).

Finally, I tested to see if the current inhibition during hypoosmotic perfusion caused by carbenoxolone could be reversed by adding ATP or thrombin to the perfusion solution. For these experiments, the voltage step protocol was performed with 20 mV steps between -90 mV to +130 mV. Data were taken at +130 mV. Previous studies showed addition of thrombin (Figure 3) did increase chloride conductance during isoosmotic CsCl treatments but added ATP (Figure 4) did not affect VRAC during isoosmotic exposure. ATP or thrombin was added to the hypoosmotic CsCl bath solution containing 100 μM carbenoxolone (Figure 5B). I found no recovery of the swelling-activated current when ATP was present in the 200 mOsm solution containing carbenoxolone.

However, adding 5 U/mL thrombin to the hypooosmotic solution containing carbenoxolone resulted in a partial recovery of the hypoosmotic current ($p=0.0109$).


A
Isoosmotic CsCl
Hypoosmotic CsCl
Swelling-activated Current
Current Density (nA/pF)
200
150
100
50
0
-50
*
*
*
*
No Drug
Apyrase
Carbenoxolone
Meclofenamate


B
Isoosmotic CsCl
Hypoosmotic CsCl
Swelling-activated Current
Current Density (nA/pF)
200
150
100
50
0
-50
*
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No Drug
Cx
Cx + ATP
Cx + Thrombin

Figure 5: The effect of limiting hemi-channel ATP release on VRAC activation. Current was measured while cells were perfused in isoosmotic (290 mOsm) CsCl solution and hypoosmotic (200 mOsm) CsCl bath solution. For control recordings (n=11), current was measured while cells were perfused in isoosmotic 290 mOsm CsCl solution and hypoosmotic 200 mOsm CsCl bath solution without added drug. Current density was calculated using the capacitance of each cell and an average was taken at +130 mV readings. (A) 5 U/mL apyrase was added to the 200 mOsm CsCl bath solution (n=5). 100 µM carbenoxolone was added to the 200 mOsm CsCl bath solution (n=6). 100 µM meclofenamate was added to the 200 mOsm CsCl bath solution (n=3). (B) 100 µM carbenoxolone was added to the 290 mOsm and 200 mOSm bath solutions. 100 µM carbenoxolone and 100 µM ATP was added to the 200 mOsm CsCl bath solution (n=5). 100 µM carbenoxolone and 5 U/mL thrombin was added to the 200 mOsm bath solution (n=4). Data show mean values for each experiment and error bars display SEM for each set of data. The (*) in A indicates significance ($\alpha<0.05$) in comparison to the mean value measured in the same osmolality without drug. The (*) in B indicates significance ($\alpha<0.05$) in comparison to the mean value measured in the same osmolality with carbenoxolone inhibition.

V. Discussion

A. Summary of experimental approach

For decades, research has examined the channel responsible for the swelling-induced chloride current, $I_{Cl,swell}$, because this channel appears to play an important role in regulating cell volume following cellular swelling (Okada et al. 2001, Nilius & Droogmans 2003). The identity of this channel, termed the volume regulated anion channel (VRAC), has been characterized electrophysiologically, pharmacologically, and structurally. VRAC conductance consists of an outwardly-rectifying anion-selective current at depolarized potentials (Blum, Walsh & Dubyak 2009). This current is predominantly present in swollen cells and develops within the first few minutes following hypotonic challenge. Inactivation of the current occurs at hyperpolarized potentials and can vary between cell types (Nilius, Sehrer & Droogmans 1994). LRRC8A proteins form an oligomer, SWELL1, during osmotic challenges. These complexes are sufficient to form anion channels sensitive to osmotic stress (Syeda et al. 2016). Topology of this protein complex suggests the leucine-rich pore forming structure is imbedded inside the plasma membrane (Qiu et al. 2014). However, channel gating and pore formation is still not understood.

I examined VRAC activation in P2Y2 stably-transfected 1321N1 astrocytoma cells using the voltage clamp procedure. To isolate chloride conductances through the plasma membrane, cesium chloride (CsCl) was used in the extracellular bath solution and

intracellular patch solution. Assuming there is no permeability pathway for cesium ions, by using CsCl solute in the intracellular and extracellular solutions, I eliminated K^+ currents and was thus able to isolate negatively charged chloride ion movement across the plasma membrane using whole-cell patch clamp electrophysiological studies. The role of the P2Y2 receptor and downstream G-protein signaling was investigated pharmacologically.

B. Identification of VRAC in 1321N1 cells

VRAC shows an outwardly rectifying chloride conductance during cellular swelling, displays time-dependent inactivation at depolarizing membrane potentials, and is blocked by DCPIB (Decher et al. 2001, Walsh & Dubyak 2009). In this study, P2Y2 transfected 1321N1 cells displayed such an outwardly rectifying chloride current with time-dependent inactivation at depolarized potentials during hypoosmotic exposure. This chloride current was not active under isoosmotic conditions. Additionally, a smaller chloride current similar in time and voltage dependence was observed when cells were treated with 250 mOsm hypoosmotic CsCl solution suggesting the magnitude of $I_{Cl,swell}$ activation is related to the degree of cell swelling. Finally, this current was significantly inhibited in the presence of DCPIB. Thus, I conclude VRAC is present in these tumor cells and VRAC is the channel responsible for $I_{Cl,swell}$ during cell swelling of the 1321N1 cells.

C. GPCRs are necessary for VRAC activation

P2Y2R purinergic signaling via ATP stimulation may play a role in modulating the RVD response associated with cell swelling (Dezaki, Tsumura, Maeno & Okada 2000). Previous studies using 1321N1 astrocytoma cells found the native cell type possesses no function purinergic receptors, does not activate chloride currents, and does not show a RVD (Wenker & Olson 2009). I used 1321N1 stably transfected with the human P2Y2 receptor in these experiments to determine the role purinergic signaling through this G-protein couple receptor may play in VRAC activation. These receptors have been shown to enhance volume-activated currents following ATP release in other cell types (Wang, Mehta & Rose 1995). This volume-activated current is not observed in isoosmotic conditions (Jackson & Strange 1995). To determine if G-protein coupled receptors are involved in VRAC activation in these P2Y2 transfected cells, I used the G-protein inhibitors pertussis toxin (PTX) and suramin. Pertussis toxin is an A-B exotoxin that inhibits G_0 and G_i proteins via ADP-ribosylation. PTX acts by preventing G-proteins from interacting with their GPCR by locking the α subunit in an inactive state (Mangmool & Kurose 2011). When PTX was added to the pipette solution during hypoosmotic exposure VRAC activation was significantly reduced

Similarly, when suramin, a P2Y receptor antagonist, was added to the bath solution, a significant inhibition of the swelling-induced chloride current was observed. These results suggest GPCR is necessary for VRAC activation.

D. PAR activates an osmotically-sensitive chloride current

Previous studies found PAR activation in 1321N1 cells resulted in an increase in ATP release during isoosmotic and hypoosmotic conditions (Joseph, Buchakjian & Dubyak 2003). In bovine artery endothelial cells, activation of PAR led to an increase in $I_{Cl,swell}$ (Manolopoulos, Prenen, Droogmans & Nilius 1997). I found the application of 5 U/mL thrombin enhanced the current activation during cell swelling along with the current activation during isoosmotic CsCl exposure in 1321N1 astrocytoma cells. These results suggest PAR may activate an osmotically-sensitive chloride current, but this chloride efflux is not through the volume regulated anion channel.

E. ATP release through hemi-channels is not necessary for VRAC activation

Exogenously applied ATP enhances swelling-induced chloride current during hypoosmotic exposure of primary cultured astrocytes (Darby et al. 2003). However, since swelling itself may cause ATP release it is not clear whether ATP is necessary for channel activation. Therefore, I examined the effect of additional extracellular ATP on VRAC activation as well as the effect of removing ATP from the extracellular fluid or inhibiting ATP release pathways. Apyrase hydrolyzes ATP to ADP and AMP species which are less powerful agonists of the P2Y2 receptor while carbenoxolone and meclofenamate block ATP release through gap junction hemi-channels. When 100 μM ATP was added to the cesium chloride bath solution there was no significant change in VRAC activity during isoosmotic or hypoosmotic exposure. Thus, extracellular ATP is not sufficient to activate this channel. Research has suggested ATP release during cell

swelling is required for $I_{Cl,swell}$ (Darby et al. 2003). To limit the extracellular ATP concentration, I added 5 U/mL apyrase, to the bath solution. This resulted in a small reduction in VRAC activation during hypoosmotic exposure which was significant, findings similar to those seen in Intestine407 cells where apyrase inhibited RVD (Dezaki, Tsumura, Maeno & Okada 2000). The results from these experiments suggest extracellular ATP is involved in activating VRAC but only in cells swollen by hypoosmotic exposure.

Meclofenamate, a connexin hemi-channel inhibitor, had no significant effect on VRAC activity during hypoosmotic exposure, however, carbenoxolone, a selective gap junction blocker did demonstrate a significant inhibitory effect on VRAC conductance. These results are similar to those found by Thompson, Zhou and MacVicar in hippocampal neurons (Thompson, Zhou & MacVicar 2006). However, subsequent to performing these experiments other investigators reported that carbenoxolone directly inhibits VRAC (Benfenati et al. 2009). Thus, I cannot conclude that carbenoxolone is blocking VRAC activation by reducing the release of intracellular ATP via gap junction hemi channels.

ATP and thrombin also were added to hypoosmotic perfusion solutions containing carbenoxolone. Adding ATP to the bath solution had no significant effect on VRAC activation suggesting that 100 μM extracellular ATP cannot reverse the inhibition due to carbenoxolone. However adding 5 U/mL of thrombin to cells perfused with carbenoxolone did show an increased chloride current measured in hypoosmotic conditions when compared to cells perfused with carbenoxolone inhibition. Because carbenoxolone has been found to block VRAC, this increase by thrombin in the chloride

current measured in hypoosmotic conditions is through a separate chloride conductance pathway from VRAC.

F. Conclusion

Previous research has shown chloride current is increased during hypoosmotic exposure (Zhang & Jacob 1997). VRAC is the channel which causes this increased chloride current, $I_{Cl,swell}$. However, the mechanisms of activation, channel gating, and plasma membrane insertion of channel proteins are not entirely understood. Using voltage clamp procedures during hypoosmotic exposure, I isolated the chloride current associated with VRAC in P2Y2 transfected 1321N1 cells.

Since decreasing the extracellular ATP concentration reduces the magnitude of VRAC activation, I conclude that purinergic signaling plays a role in activating this channel. However, exogenous ATP does not activate VRAC in isoosmotic conditions or enhance the chloride current during hypoosmotic exposure. Adding carbenoxolone to the bath solution did show an inhibitory effect on VRAC in the tumor cells which agrees with the findings by Darby et al. (2003); however, it is unclear if carbenoxolone acts on VRAC directly or affects VRAC by limiting ATP release through hemi-channels. Future experiments may be considered to determine if higher concentrations of ATP have any effect on channel activation in this cell type.

The enhancement of hypoosmotic activated anion current by thrombin suggests PAR may affect osmotically-sensitive chloride current but this current is not through VRAC. Thus, PAR may activate a separate chloride conductance pathway during cell swelling.

I have found that GPCR is involved in channel activation. Inhibition of P2Y2R or the G-protein signaling pathway during hypoosmotic exposure was found to inhibit the anion current associated with VRAC. However it is unclear what role GPCR is involved in channel activation, gating, and/or pore formation.

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