Receptor Regulation of Osmolyte Homeostasis in Neural Cells in Response to Hyposmotic Stress

by

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A dissertation submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy
(Pharmacology)
in The University of Michigan
2010

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Acknowledgements

It is a pleasure to thank those who made this thesis possible. I would like to thank my beautiful wife as well as my mother and father for their love and support throughout the years. I'd also like to express gratitude to my advisor, Dr. Stephen Fisher, for his extensive help and guidance. I am also grateful to Drs. Anne Heacock, Margaret Gnegy, Helen Baghdoyan, Richard Keep, and John Traynor for their contributions to this work.

Preface

Some of the data presented in this thesis have been previously published. Chapters 2, 3, and 4 were published in the Journal of Pharmacology and Experimental Therapeutics ((2008) 325: 457-465), the Journal of Neurochemistry ((2009) 108: 437-449), and the Journal of Pharmacology and Experimental Therapeutics ((2010) 333: 297-309) respecitvely. The appendix has been published in the Journal of Pharmacology and Experimental Therapeutics ((2006) 319: 963-971).

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List of Abbreviations

8-CPT-cAMP	8-(4-Chlorophenylthio)-cAMP
AA	Amino acid
ADP	Adenosine diphosphate
AM	Acetoxymethyl ester
AMPA	α-Amino-3-hydroxyl-5-methyl-4-isoxazole-propionate
ANOVA	Analysis of varience
ATP	Adenosine triphosphate
BAPTA	1,2-Bis(o-aminophenoxy)ethane-N,N,N',N'-tetracetic acid
BBB	Blood-brain barrier
BIM	Bisindolylmaleimide
$[Ca^{2+}]_i$	Concentration of cytosolic Ca^{2+}
CaM	Calmodulin
CaMKII	Ca^{2+} / calmodulin dependent protein kinase II
cAMP	Cyclic adenosine monophosphate
ClC	Chloride channel
CNS	Central nervous system
Cr	Creatine
DCPIB	4-[(2-Butyl-6,7-dichoro-2-cyclopentyl-2,3-dihydro-1-oxo-1H-inden-5-yl)oxy]butanoic acid

DDF	1,9-Dideoxy-forskolin
DHK	Dihydrokainate
DIDS	4-Diisothiocyano-2-stillbene-disulfonic acid
DIOA	R-(+)-[(2-n-butyl-6,7-dichloro-2-cyclopentenyl-2,3-dihydro-1-oxo-1H-inden-5-yl)oxy]acetic acid
DMEM	Dulbecco's modified Eagles's medium
DMSO	Dimethyl sulphoxide
EAAT	Excitatory amino acid transporter
EEA1	Early endosomal antigen 1
EGTA	Ethylene glycol tetraacetic acid
EP	Prostaglandin receptor
ERK	Extracellular signal-regulated kinases
GABA	γ-Aminobutyric acid
Gö6983	2-[1-(3-Dimethyl-aminopropyl)-5-methoxyindol-3-yl]-3-(1H-indol-3-yl)maleimide
GPC	Glycerophosphorylcholine
GPCR	G-protein-coupled receptor
GTP	Guanosine triphosphate
HEPES	N-[2 Hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]
IBMX	3-Isobutyl-1-methylxanthine
$\mathbf{I_{Cl,swell}}$	Outwardly rectifying Cl^- current activated by hypotonicity
IP	Inositol phosphate
ISF	Interstitial fluid

Jak-2	Janus kinase 2
KCC	K^+-Cl^- cotransporter
L3HA	L-(-)-threo-3-hydroxyaspartic acid
LβBA	L-β-threo-benzyl-aspartate
LPA	Lysophosphatidic acid
mAChR	Muscarinic cholinergic receptor
MAPK	Mitogen-activated protein kinase
mGluR	Metabotropic glutamate receptor
Myo-I	Myo-inositol
NAA	N-acetyl aspartate
NKCC	Na^+-K^+-$2Cl^-$ cotransporter
NMDA	N-Methyl-D-aspartic acid
NPPB	5-Nitro,2(3-phenylpropylamino) benzoic acid
OPA	o-phthaldialdehyde
OsM	Osmoles / L
Oxo-M	Oxotremorine-M
P13K	Phosphatidylinositol 3-kinase
PAR	Protease-activated receptor
PCr	Phosphocreatine
PEA	Phosphatidylethanolamine
PG	Prostaglandin
PI	Phosphoinositide
PKA	Protein kinase A

PKB	Protein kinase B
PKC	Protein kinase C
PLC	Phospholipase C
PMA	Phorbol 12-myristate 13-acetate
QNB	Quinuclidinyl benzilate
rtPCR	Reverse transcription polymerase chain reaction
RVD	Regulatory volume decrease
RVI	Regulatory volume increase
S1P	Sphingosine-1-phosphate
siRNA	Small interfering RNA
TauT	Taurine transporter
TBOA	DL-threo-β-benzyloxyaspartic acid
TCA	Trichloroacetic acid
TK	Tyrosine kinase
TRP	Transient receptor potential
TRPV	Transient receptor potential vanilloid
VEGF	Vascular endothelial growth factor
VRAC	Volume-regulated anion channel
VSOAC	Volume-sensitive organic osmolyte and anion channel

Abstract

Cell swelling can have profound deleterious effects in the brain and is observed to occur during several pathological conditions. Upon swelling, cells regulate their volume through the extrusion of various osmolytes. Prior studies in both chronically hyponatremic rat brains and cultures of primary astrocytes suggest that individual osmolytes are differentially utilized during cell volume regulation. In these models organic osmolytes are depleted from cells, whereas inorganic osmolytes are comparatively retained. Although selective osmolyte depletion has been appreciated for a number of years, the mechanism whereby this occurs has remained unknown. Activation of certain G-protein-coupled receptors, including muscarinic cholinergic receptors (mAChRs), has been demonstrated to non-selectively stimulate the release of both organic and inorganic osmolytes. However, the ability of the same receptors to regulate osmolyte influx has not been examined. I discovered that hypotonicity and receptor activation stimulated both the efflux and influx of K^+ (monitored with $^{86}Rb^+$) in SH-SY5Y cells and cultures of primary rat astrocytes. Furthermore, in SH-SY5Y cells, these fluxes (mediated primarily by K^+ channels for efflux, and the Na^+/K^+ATPase and NKCC transporters for influx) were found to be of a similar magnitude so as to permit the retention of intracellular K^+ during physiologically-relevant reductions in osmolarity. In contrast, taurine uptake

(mediated via the taurine transporter) was inhibited by hypotonicity and mAChR activation in SH-SY5Y cells and cultured astrocytes. This process, when combined with increased taurine efflux, would promote taurine depletion. I also demonstrated that activation of mAChRs on SH-SY5Y cells, under isotonic conditions, resulted in an increased glutamate uptake (monitored as ^{3}H-D-aspartate) and redistribution of the excitatory amino acid transporter 3 (EAAT3) to the plasma membrane. However, hypotonicity inhibited mAChR-mediated glutamate uptake and disrupted EAAT3 trafficking. Such a process may permit glutamate to be conserved within cells during small reductions in osmolarity, whereas depletion would occur under more hyposmotic conditions. Together, these findings suggest that GPCR-mediated regulation of osmolyte influx represents a potential mechanism whereby the selective depletion or retention of osmolytes is mediated.

Chapter 1

INTRODUCTION

Fundamentals of cell volume regulation

Maintenance of constant cell volume is necessary for the survival and proper function of all cells, ranging from single-celled organisms to glia and neurons in the mammalian central nervous system (CNS). Changes in cell volume can lead to changes in intracellular ion and protein concentrations which can in turn affect membrane potential and macromolecular crowding with deleterious effects on cellular function. Cell swelling has been demonstrated to alter cellular excitability, proliferation, metabolism, and contractility as well as affect receptor recycling and hormone/transmitter release (Lang et al., 1998a). Increases in cell volume are particularly detrimental to the CNS due to the physical restrictions of the skull. In the brain small changes in cell volume can alter the spatial relationship between neurons and glia leading to altered excitability, while large increases in volume can increase intracranial pressure, impairing blood flow and causing anoxia and ischemia (Sykova, 2004). Given its fundamental importance, cell volume

conservation is aggressively regulated and homeostatic mechanisms that mediate this regulation can be found in nearly all organisms regardless of their evolutionary origin.

All animal cells are permeable to water and movement of water across the plasma membrane of animal cells is primarily mediated by osmotic pressure. Under steady-state conditions H_2O is in thermodynamic equilibrium across the plasma membrane, meaning that the osmolarity of the intracellular milieu is equal to that of the extracellular fluid (Strange, 2004). As such, any perturbations in this equilibrium via changes in the osmolarity of the intracellular, or extracellular, compartments will cause concurrent movement of water across the plasma membrane. Accordingly, etiologies of cell swelling can be broadly classified as hypotonic swelling (that which arises as a result of a reduction in the extracellular osmolarity), or isotonic swelling (which arises due to perturbations in the maintenance of intracellular ion concentrations). In the case of hypotonic swelling, the reduced osmolarity of the extracellular fluid creates an osmotic gradient that causes water to flow into the cell and induce cell swelling. Upon swelling, cells initiate a process known as regulatory volume decrease (RVD) during which the cells actively extrude various osmolytes (defined as any substance that contributes to the osmolarity of a solution) into the extracellular fluid, bringing with them osmotically obligated water allowing cell volume to return to normal (Fig. 1.1). Quantitatively major osmolytes that are used in mammalian cells to restore cell volume upon swelling include inorganic molecules such as K^+ and Cl^- as well as organic molecules such as taurine, glutamate, and inositol.

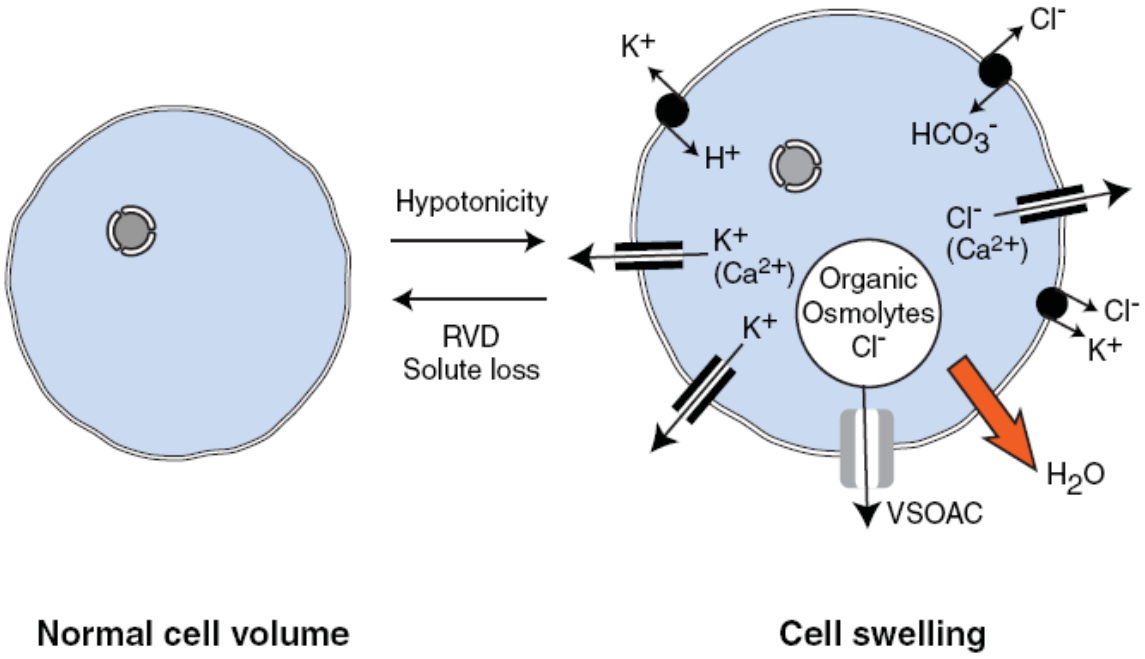

Figure 1.1 Schematic of regulatory volume decrease (RVD) in response to hypotonic swelling. Upon exposure to hypotonicity cells swell via osmosis and then extrude K^+, Cl^-, and organic osmolytes (i.e. taurine, glutamate and myo-inositol) via various channels and transporters. As these osmolytes cross the plasma membrane they bring with them osmotically-obligated water allowing cell volume to return to normal. (VSOAC: Volume-sensitive organic osmolyte and anion channel)

Conversely, when exposed to a hypertonic environment, cells shrink and undergo regulatory volume increase (RVI) during which osmolytes are either transported into the cell or synthesized to return cell volume to normal. While the exact osmolytes and molecular mechanisms whereby osmolyte fluxes are activated or mediated may differ, the goal of RVD to cause a net reduction in intracellular osmolytes, and of RVI to cause a net accumulation of osmolytes, is essentially conserved from species to species throughout evolution.

Even when extracellular osmolarity is constant cell swelling can arise as a consequence of changes in intracellular ion / osmolyte distribution. Isotonic cell swelling can occur even in the absence of overt pathology in neural cells as a result of neurotransmission, intense neuronal discharge, or upon exposure to ethanol (Lux et al., 1986; Holthoff and Witte, 1996; Nikodemova et al., 1997; Darquie et al., 2001). Several pathologies can also induce cellular edema (sometimes referred to as cytotoxic edema) in which cell volume is increased. These pathologies include stroke, traumatic head injury, acute mountain sickness, and other conditions which induce periods of ischemia / hypoxia as well as exposure to some neurotoxins (Houston, 1989; Liang et al., 2007). The mechanisms whereby cell swelling is induced during cytotoxic edema are thought to occur primarily via reductions in cellular ATP. Na^+/K^+ ATPase is an ATP-dependent transporter that exchanges 2 intracellular Na^+ ions for 3 extracellular K^+ ions, a process that is vital to maintaining cell volume as well as cell membrane potential. Reductions in oxidative phosphorylation and concurrent reductions in cellular ATP reduce Na^+/K^+ ATPase activity, causing increases in intracellular Na^+ which drives Cl^- influx that

subsequently results in an increase in the osmolarity of the intracellular compartment and cell swelling. This latter process can be exacerbated by increases in the extracellular K^+ concentration which stimulates both KCl uptake and voltage sensitive Ca^{2+} influx, which can have several consequences including excitatory amino acid release. Increased cellular hydration of CO_2 has also been demonstrated to induce NaCl influx via HCO_3^- and H^+ exchangers respectively (Kimelberg, 1995). Whereas isotonic and hypotonic cell swelling differ in etiology they may initiate similar volume-sensitive mechanisms such as activation of volume sensitive Cl^- channels and organic osmolyte efflux pathways (Inoue and Okada, 2007; Pasantes-Morales and Cruz-Rangel, 2009).

Hypotonic swelling; Hyponatremia

Although most cells possess cellular volume regulatory mechanisms that allow them to adapt to changes in extracellular osmolarity, mammals also actively regulate and maintain the osmolarity of their extracellular fluid. Some organisms (i.e. snails, trout, and sharks) are referred to as osmoconformers because they do not regulate, but simply adopt, the osmolarity of their extracellular environment (Roubos and Moorer-Van Delft, 1976; Seidelin et al., 2000; Harms et al., 2002). Humans and all other mammals, however, regulate the osmolarity of their extracellular fluid and are thus referred to as osmoregulators. In humans, the steady state of plasma osmolarity is ~290mOsM and this value is rigorously maintained by changes in salt appetite and thirst by the CNS as well as changes in natriuresis and

diuresis in the renal system (Zerbe and Robertson, 1983). This regulation is mediated primarily via osmoreceptors which are present both peripherally and centrally. Peripheral osmoreceptors are present in the alimentary tract, oropharyngeal cavity, splanchic mesentery, hepatic portal vein, and liver. Changes in osmolarity at these sites (which correlate to the sites of water and salt absorption) are relayed via the vagus nerve and activate anticipatory responses that allow plasma osmolarity to be maintained in the face of water or salt ingestion. Central osmoreceptors are found in the nucleus tractus solarius, paraventricular nucleus, and supraoptic nucleus as well as in certain circumventricular organs, which are outside of the blood brain barrier, such as the organum vasculosum laminae terminalis, neurohypophesis, and subfornical organ (Bourque, 2008). These central osmoreceptors are specialized neurons which change their firing rate and pattern in response to changes in cell volume, an effect that is mediated, at least in some cases, by transient receptor potential vanilloid (TRPV) receptors (Liedtke, 2007). In both the paraventricular and supraoptic nuclei, exposure of such osmosensitive neurons to hyposmolarity inhibits firing and attenuates concomitant vasopressin release in the neurohypophysis. By decreasing basal vasopressin release these osmoreceptors increase renal diuresis allowing the return of plasma osmolarity back to normal. In addition to effects on diuresis there is evidence to suggest that these central osmoreceptors can also affect salt and water appetite as well as natriuresis (Bourque, 2008). These osmosensitive neurons are unique not only in their ability to regulate plasma osmolarity but also lack cell volume regulatory mechanisms and do not correct their volume in the face of osmotically-induced swelling or shrinking

(Zhang and Bourque, 2003). This allows for these neurons to maintain sustained responses to plasma osmolarity that are not attenuated upon volume correction.

Despite the presence of intricate regulatory systems, plasma osmolarity does exhibit some variations under physiological conditions. In dehydrated individuals, drinking 850 ml of H_2O (equivalent to about two glasses) will cause a ~ 2% decrease in plasma osmolarity within a half hour (Geelen et al., 1984). Several pathological conditions can also affect plasma osmolarity including hyponatremia, the most prevalent electrolyte disorder encountered in the clinic, which is estimated to occur in 15-20% of hospitalized patients (Flear et al., 1981; Hawkins, 2003).

Hyponatremia is defined as a reduction in the serum Na^+ concentration from its normal value of ~ 145 mEq/L to ≤ 136 mEq/L. This magnitude of a reduction in serum Na^+ concentration causes systemic hypotonic cell swelling (Multz, 2007). The etiologies of hyponatremia are diverse and include over-hydration which is commonly encountered in marathon runners as well as during schizophrenic polydipsia. Alternatively, congestive heart failure, syndrome of inappropriate secretion of vasopressin, liver cirrhosis, and use of selective serotonin reuptake inhibitors or thiazide diuretics can also lead to hyponatremia (Haussinger et al., 1994; Pasantes-Morales et al., 2002b; Rosner, 2004). Although hyponatremia can arise from multiple etiologies, 97% of hyponatremic patients with plasma Na^+ concentrations ≤ 130 mEq/L were reported to have increased plasma vasopressin levels, suggesting that dysregulation of vasopressin release is often an underlying cause of hyponatremia (Anderson et al., 1985).

Hyponatremia is generally associated with poor clinical outcomes as hyponatremic patients have a fatality rate that is 6-70 times that of non-hyponatremic patients (Bhardwaj, 2006). The majority of hyponatremic patients are non-symptomatic as the body is able to adapt to minor and gradual reductions in plasma osmolarity. In contrast, patients experiencing either severe chronic reductions in osmolarity or alternatively who experience an acute onset of hyponatremia, are often symptomatic, with larger reductions in plasma osmolarity correlating to more deleterious manifestations (Multz, 2007). Whereas the prevalence of hyponatremia is similar in both women and men, women have a much poorer prognosis in terms of death and brain damage (Fraser and Swanson, 1994; Ayus et al., 2008). Of all the organs in the body the brain is the most sensitive to changes in osmolarity and accordingly most of the symptoms observed in hyponatremic patients are neurological in nature. When the plasma osmolarity is between 125 and 135 mEq/L, patients often report mild nausea and headaches whereas below 120 mEq/L (around a 20% reduction in plasma osmolarity) seizures, coma, and permanent brain damage or death may occur (Bhardwaj, 2006).

Treatment of hyponatremia traditionally consists of fluid restriction, administration of hypertonic saline, and prescription of vasopressin antagonists, all with the goal of restoring plasma osmolarity back to normal (Palm et al., 2006). Paradoxically, an overly rapid correction of plasma osmolarity can have adverse consequences. As mentioned above, cells respond to hyposmolarity by activating a process known as RVD whereby osmolytes are released so as to equilibrate the osmolarity of their intracellular milieu to that of the plasma. Accordingly, cells that

have been exposed to hypotonic plasma will have a reduced intracellular osmolarity and ‘isotonic’ 290 mOsM plasma will be perceived as hypertonic, thereby causing cell shrinkage which can lead to adverse effects such as brain dehydration and central pontine myelenosis (McManus et al., 1995; Sterns and Silver, 2006).

The brain is relatively unique in that its volume is confined to a limited space by the physical restrictions imposed by the skull. As a consequence, the brain has little room to swell and small changes in brain volume may lead to drastic changes in the spatial relationship between cells leading to altered neurotransmission as well as altered blood flow. Accordingly, it has long been hypothesized that changes in total brain volume are responsible for the symptoms observed in hyponatremic patients (Gullans and Verbalis, 1993). During episodes of hyponatremia, the reduction in plasma osmolarity results in an influx of water into the brain through the blood-brain barrier and into neural cells across their plasma membranes. However, the increases in brain water are less than expected from a perfect osmometer (Verbalis & Drutarosky, 1988). The extracellular space is estimated to occupy approximately 20% of the brain volume and increases in brain volume will affect this parameter leading to changes in both the lateral diffusion of neurotransmitters and the extracellular concentration of neurotransmitter per quantum released (Sykova et al., 2004; Thorne and Nicholson, 2006). Acute reductions in plasma osmolarity (10-20%) increase brain volume by 5-7%, a magnitude which can lead to compression of blood vessels, which generates episodes of ischemia and anoxia (Pasantes-Morales et al., 2002b). As intracranial pressure increases and brain volume increases to 110% of normal, the brain parenchyma can be displaced through the foramen

magnum, which can lead to death via cardiac or respiratory arrest (Sterns and Silver, 2006).

Increases in brain volume occur as a result of the osmotic movement of water from the plasma across the blood brain barrier (BBB). Water permeability to a lipid bilayer membrane is around 1 μm s^{-1} and can increase by up to 10 fold in the presence of membrane spanning proteins such as channels and transporters (Fettiplace and Haydon, 1980). Accordingly, the kinetics of water transport is generally of a sufficient magnitude such as not to be a rate limiting step in osmotic swelling at the cellular level. However, membrane water permeability can be enhanced even further (up to 100 μm s^{-1}) by the expression of water channels known as aquaporins (Yang and Verkman, 1997). Several studies have suggested that aquaporin-mediated water transport plays a significant role in determining changes in brain volume during acute hyponatremia. Several aquaporins are found in the brain including aquaporins 1, 4, and 9. Aquaporin 4 is locally expressed in perivascular astrocytes, cells that are near the BBB interface. Knock downs of aquaporin 4 or various proteins that act to localize aquaporin 4 to astrocytic endfeet have been demonstrated to render mice resistant to hyposmotic stress (Manley et al., 2000; Vajda et al., 2002; Amiry-Moghaddam et al., 2004). Conversely, glia-specific transgenic overexpression of aquaporin 4 has been demonstrated to potentiate hyposmotic-induced edema and associated mortality (Yang et al., 2008). These effects are thought to be primarily mediated via alterations in the kinetics with which water can cross the BBB and thus modulate brain volume. Aquaporin 4 null mice do not exhibit any basal edema suggesting that the role of these proteins is

primarily to mediate water transport into or out of the brain in response to pathological changes in brain volume (Amiry-Moghaddam and Ottersen, 2003).

Brain volume regulation

Due to the presence of regulatory mechanisms, brain volume does not change in response to systemic hyponatremia as much as one would expect on the basis of ideal osmotic behavior. In fact, brain volume during chronic hyponatremia was observed not to be significantly different from that observed in control animals (Verbalis and Drutarosky, 1988). This observation reflects the presence of homeostatic response mechanisms that maintain brain osmolarity and volume. During hyponatremia water is osmotically driven across the BBB reducing the osmolarity of the ISF and causing concurrent cell swelling. Accordingly, the total volume of the brain increases due to increases in both cellular and ISF volume. Upon swelling, the brain adaptively responds by extruding various osmolytes which then bring with them osmotically obligated water and return brain volume back to normal.

The osmolytes lost from chronically hyponatremic brains and their contribution to brain volume maintenance are depicted in Fig. 1.2. Within a half hour of the onset of hyponatremia *in vivo*, the amount of NaCl in the brain is significantly reduced. The nature of this NaCl loss does not reflect an increase in NaCl transport across the BBB, but instead seems to involve movement of NaCl into the cerebral spinal fluid via increased hydrostatic pressure. As NaCl is transported

out into the ISF, and eventually out into the systemic circulation, it brings with it water thus reducing the volume of the ISF. Loss of NaCl from the brain is rapid, but not sustained, as reductions in brain NaCl are maximal 3 hours after onset of hyponatremia (Melton et al., 1987).

In addition to the initial and rapid loss of NaCl from the ISF, a sustained response occurs whereby intracellular osmolytes (primarily K^+, Cl^-, taurine, myo-inositol, and glutamate) are removed from the brain. The mechanism whereby these intracellular osmolytes are released into the ISF is referred to as regulatory volume decrease (RVD). As these intracellular osmolytes cross the plasma membrane they bring with them osmotically obligated water, returning cell volume to normal and making the osmolytes available for clearance from the ISF and brain. Both neurons and astrocytes, when maintained in culture, are observed to swell in response to hyposmotic stress. Such hypotonic swelling has been demonstrated to induce Cl^- currents, osmolyte release, and / or RVD in a wide variety of neural cells including Purkinje cells (Nagelhus et al., 1993), sympathetic ganglia (Leaney et al., 1997), cerebellar granule cells (Moran et al., 1997; Morales-Mulia et al., 2001), mouse sensory trigeminal neurons (Viana et al., 2001), NT2-N neurons (Novak et al., 2000), neuroblastoma (Altamirano et al., 1998; Loveday et al., 2003), and primary cultures of hippocampal and cortical neurons (Li and Olson, 2004; Inoue et al., 2005). Glial cells, which constitute the majority of cells in the brain, also have been demonstrated to undergo RVD in a variety of cultured cell types including 1321N1 astrocytoma (Cheema et al., 2005), D54-MG glioma (Ernest et al., 2005), and C6 glioma cells (Lohr and Yohe, 1994) as well as in primary cultures of pituicytes

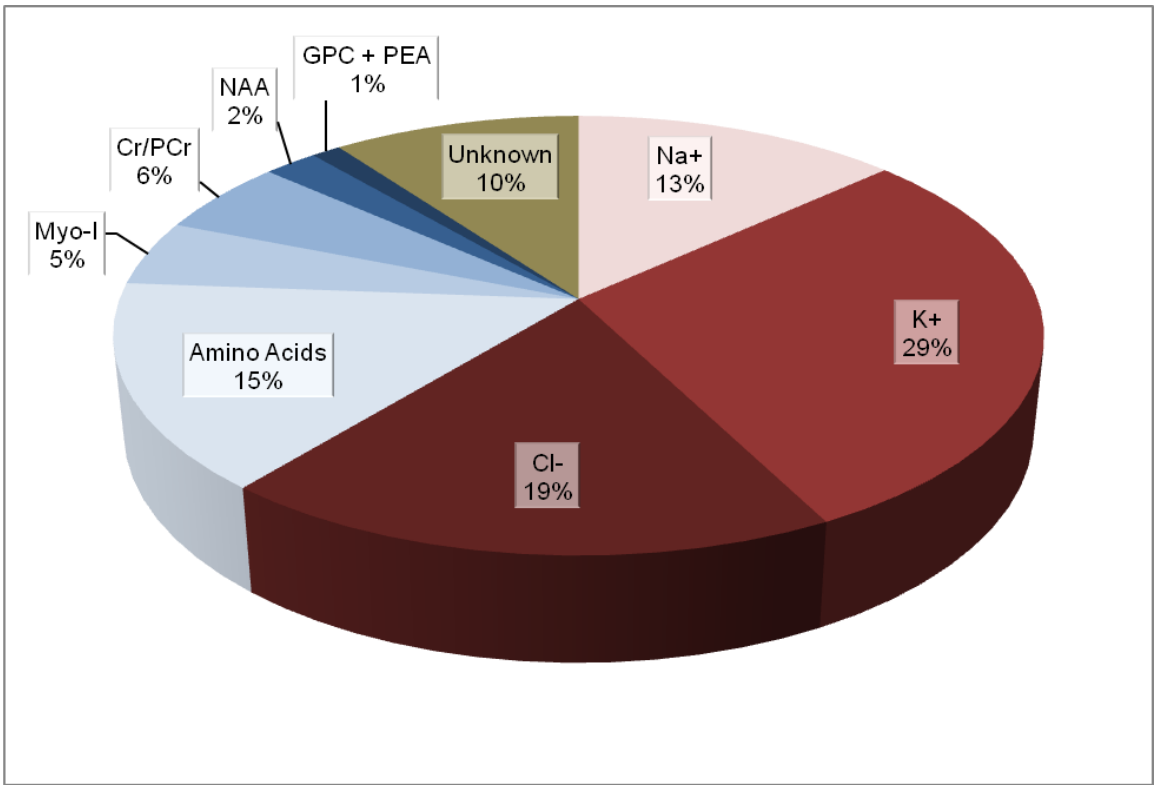

Figure 1.2 Relative contribution of various osmolytes to brain volume regulation. Major inorganic osmolytes (shades of red) and organic osmolytes (shades of blue) involved in adaptations of the brain to chronic hyponatremia. GPC: glycerophosphorylcholine, PEA: phosphatidylethanolamine, NAA: *N*-acetyl aspartate, Cr/PCr: creatine/phosphocreatine; Myo-I: myo-inositol, AA: Amino acids (Adapted from Pasantes-Morales et al., 2002a).

(Rosso et al., 2004; Pierson et al., 2007) and cortical astrocytes (Mongin and Kimelberg, 2002; Mongin and Kimelberg, 2005; Pasantes-Morales et al., 2006b; Ramos-Mandujano et al., 2007). Organic osmolyte release, a hallmark of RVD, has also been observed in more intact tissue preparations such as brain slices, isolated nerve preparations and biopsies of gliomas from human patients (Tuz et al., 2001; Bothwell et al., 2002; Ernest et al., 2005).

Inorganic vs. Organic Osmolytes

All of the osmolytes utilized by cells during RVD can be categorized as either inorganic or organic. The major inorganic osmolytes that are utilized during RVD in the human brain are K^+ and Cl^- and the primary mechanism whereby K^+ and Cl^- efflux is mediated is via activation of volume-sensitive channels. K^+ and Cl^- combine to account for a large portion (~50%) of the change in brain osmolarity that occurs during chronic hyponatremia (Fig. 1.2). All of the K^+, and some of the Cl^-, depleted from the hyponatremic brain is thought to be lost as a result of RVD, while the remainder of the Cl^-, and all of the Na^+, are thought to occur independently of RVD, during the initial removal of ISF from the brain (Melton et al., 1987). The significance of K^+ and Cl^- to RVD is due, in large part, to their high intracellular concentrations, particularly for K^+ which is extremely concentrated (~150 mM) intracellularly in the brain. Despite their large contribution to brain volume regulation, the loss of K^+ and Cl^- from brain cells can be detrimental to the cell. Alterations in the intracellular concentration of K^+ and Cl^- will affect several plasma membrane transporters whose

operation is dependent on the energy stored in the electrochemical gradients of these ions. In addition, K^+ and Cl^- fluxes can have direct effects on intracellular ionic strength as well as cell membrane potential and concurrent excitability (Lang et al., 1998a).

Due to the potentially deleterious effects that could arise from substantial changes in inorganic osmolyte concentrations it is not surprising that brain cells have adapted to use small organic molecules that have been considered to act as 'compatible' or 'non-perturbing' osmolytes. Organic osmolytes contribute ~35% to the adaptation of the hyponatremic rat brain to chronic hyposmolarity (Fig. 1.2), and proton magnetic resonance spectroscopy studies indicate that a pronounced loss of organic osmolytes is also observed from the human brain under conditions of severe hyponatremia (Haussinger et al., 1994; Videen et al., 1995).

The organic osmolytes can be subdivided into three main classes; (i) polyols, such as myo-inositol and sorbitol; (ii) amino acids such as taurine, glutamate, *N*-acetyl aspartate, creatine, and glycine or (iii) methylamines, such as betaine, phosphatidylethanolamine, and glycerophosphorylcholine (Nilius et al., 1997; Lang et al., 1998a; Pasantes-Morales et al., 2000a). In both neural and non-neural tissues the concentrations of individual organic osmolytes change during development, with the quantitatively major organic osmolytes in the rat CNS being taurine, glutamate, myo-inositol, glutamine, and creatine (Miller et al., 2000). However, several other neuroactive amino acids or derivatives are also released from neural cells in response to hypotonic stress including GABA, glycine, aspartate, and *N*-acetyl-aspartate (Pasantes-Morales et al., 2002b), all of which are present in

mM concentrations in the CNS (McIlwain and Bachelard, 1971). Whereas organic osmolytes as a class, when compared to inorganic osmolytes, are typically regarded as inert, individual organic osmolytes differ widely with regards to the deleterious effects that can arise from their release.

The β-amino acid taurine (2-aminoethane sulfonic acid) is generally considered one of the more 'ideal' osmolytes in the brain due to its high concentration and relatively inert nature. Taurine is the third most abundant amino acid in the brain (0.5-12 mM in the CNS; Huxtable, 1989; Pow et al., 2002) and lacks a net charge (taurine is zwitterionic at physiological pH). Although taurine plays an important metabolic role in the liver via the conjugation of bile acids (Vessey, 1978), its function in the brain appears to be relatively restricted to that of an osmolyte, although reports have suggested that taurine may possess neuroprotective, anti-apoptotic, and Ca^{2+} buffering capabilities (Warskulat et al., 2007; El Idrissi and L'Amoreaux, 2008). Taurine is also a relatively inert molecule once released into the ISF of the brain. Taurine, however, is not completely inert and can regulate $GABA_A$, $GABA_B$, NMDA and strychnine-sensitive glycine receptors. This latter interaction has been demonstrated to have physiological implications in the neurohypophysis (Albrecht and Schousboe, 2005; Jia et al., 2008). Taurine is highly enriched in the astrocytes present in the neurohypophysis, and when these astrocytes are exposed to hypotonicity taurine is readily released (Pow et al., 2002; Rosso et al., 2004). Once released, taurine can then activate glycine receptors present on the terminals of nerves that originate in the supraoptic nucleus, resulting in an inhibition of vasopressin release (Hussy et al., 2001). Vasopressin, acting via V_{1A} receptors and

a rise in $[Ca^{2+}]_i$ results in a further potentiation of the volume-dependent release of taurine. Thus, the ability of vasopressin (and co-released ATP) to stimulate taurine efflux provides a powerful feedback inhibition loop that, in a paracrine fashion, inhibits further neuropeptide release under hypotonic conditions (Rosso et al., 2004). Decreased vasopressin release will increase diuresis at the kidney and act to return plasma osmolarity back to normal. Conversely, ATP co-released with vasopressin can be broken down to adenosine by ectoATPases, which can then act at A_{2B} adenosine receptors and attenuate taurine release via a cAMP-dependent mechanism, thereby providing an additional means whereby vasopressin release can be regulated (Pierson et al., 2007). In summary taurine, while not completely inert, seems to be ideally suited as an osmolyte in that its release has few detrimental effects and once released taurine can act as a paracrine signal which can help the body respond to reductions in plasma osmolarity. On the other end of the spectrum, glutamate is a quantitatively major organic osmolyte that can act at NMDA, AMPA, and metabotropic glutamate receptors and when released can cause deleterious effects such as excitotoxicity and can actually induce cell swelling in astrocytes (Chan et al., 1990).

Because the contribution of an osmolyte to brain cell volume correction is based, in large part, upon both its extra- and intracellular concentrations, these data (as depicted in Fig. 1.2) do not tell us anything about the extent to which an osmolyte is depleted during hyponatremia. When the loss of brain osmolytes is reported as a percentage reduction of that osmolyte, we see that organic osmolytes are largely depleted while inorganic osmolytes are comparatively retained in

hyponatremic rat brains (Fig. 1.3). Taurine and myo-inositol are severely depleted from the brains of chronically hyponatremic rats with taurine levels reduced by up to 95%; conversely K^+ and Cl^- are only modestly depleted by 10-20% (Thurston et al., 1989; Lien et al., 1991; Verbalis and Gullans, 1991; Massieu et al., 2004). Glutamate on the other hand, while still depleted to a greater extent than inorganic osmolytes, is not lost to the same extent as taurine and myo-inositol. This could indicate that mechanisms exist which act to selectively extrude relatively inert osmolytes from the brain (i.e. taurine and myo-inositol) while comparatively retaining those which can have detrimental effects upon release / depletion (i.e. K^+, Cl^-, and glutamate).

Swelling-Activated K^+ Efflux Pathways

During RVD, K^+ efflux can occur via K^+ channels and / or the K^+/Cl^- cotransporter. Evidence from a wide variety of tissues suggests that different channels may mediate volume-sensitive K^+ fluxes. These include large conductance (B_K), intermediate conductance (I_K), small conductance (S_K), voltage sensitive ($K_V1.3$, $K_V1.5$, $K_V4.2$, and $K_V4.3$) and two pore (TASK-2, TREK-1, and TRAAK) potassium channels in addition to the K^+ channels derived from KCNQ genes (KCNQ1, KCNQ4, KCNQ5) (Hoffmann et al., 2009). The Ca^{2+}-sensitive I_K channel has been identified as mediator of volume-sensitive K^+ efflux in GH4/C1 pituitary tumor cells (Jakab et al., 2006). However, given the breadth of volume-sensitive K^+ channels identified in other tissues, there is a paucity of information regarding which

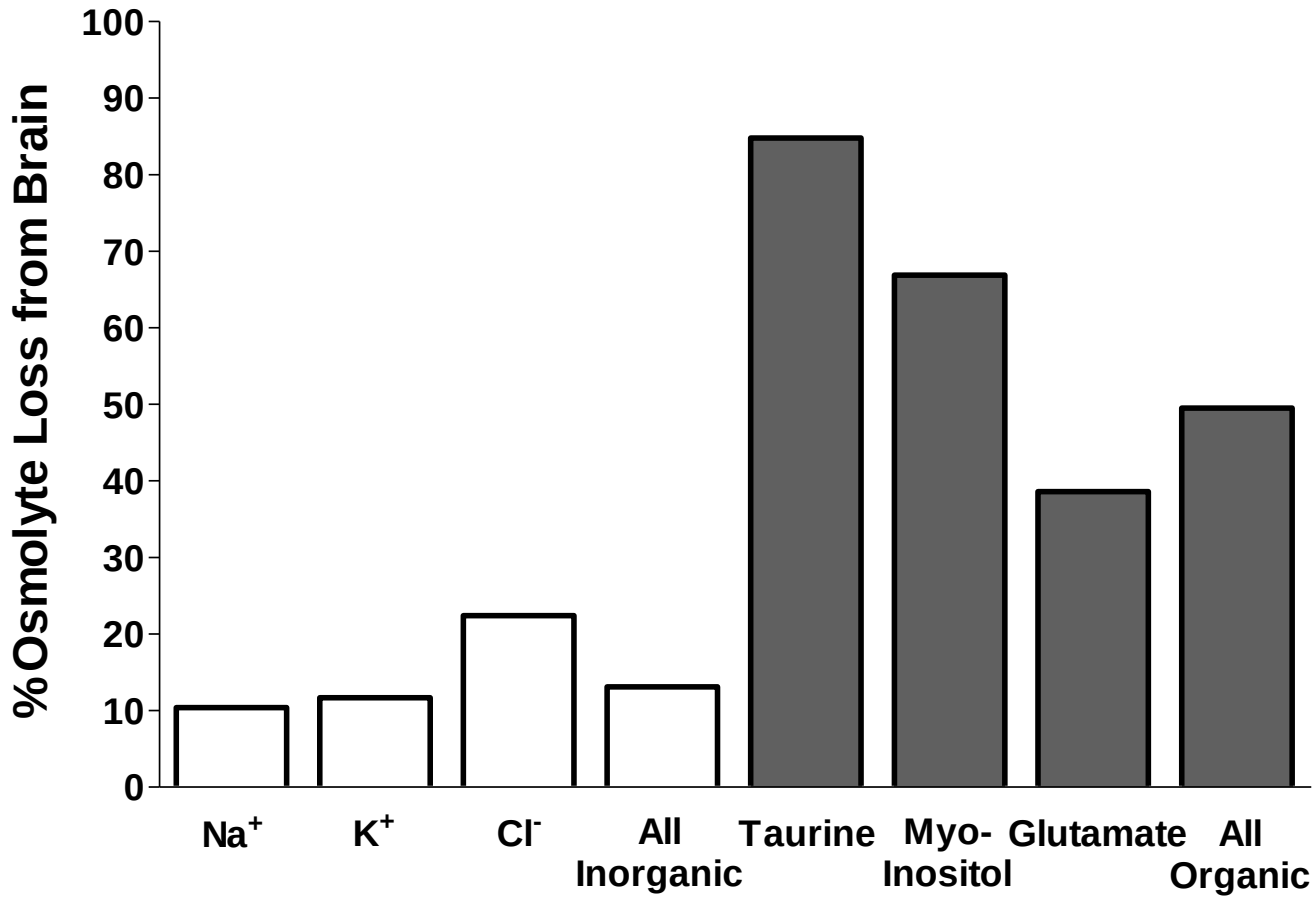

Figure 1.3 Depletion of inorganic and organic osmolytes from the brains of chronically hyponatremic rats. Inorganic osmolytes (open bars) are comparatively retained in the brain during volume regulatory processes compared to organic osmolytes (filled bars). Among the organic osmolytes taurine and myo-inositol are more severely depleted than the neurotoxic glutamate (Adapted from Pasantes-Morales et al., 2002b)

channels mediate volume-sensitive K^+ fluxes in cells found in the brain. In C6 glioma cells, distinct K^+ channels (one Ca^{2+}-sensitive, the other Ca^{2+}-insensitive) are differentially activated depending on the degree of cell swelling (Ordaz et al., 2004), a factor that must be considered when determining the physiological significance of various K^+ channels to volume regulation. If such a mechanism, whereby multiple K^+ channels are activated / inactivated depending on the extent of cell swelling, is common to other neural cell types this could complicate identification of volume-sensitive K^+ channels in these cells.

Swelling-mediated K^+ efflux can also occur via the K^+-Cl^- symporter (KCC). To date there have been 4 subtypes of KCC identified (KCC1-4) with KCC3 comprising of a short and long isoform (KCC3a and KCC3b). The KCC proteins constitute one half of the SLC12A family of transporters and mediate electroneutral transport of K^+ and Cl^- across the plasma membrane. This K^+/Cl^- cotransport can occur in either direction, but during hypotonic swelling KCCs are primarily thought to mediate K^+ and Cl^- efflux. Of the KCC isoforms, KCC1, 3a, and 4 have been identified in cultures of primary neurons while KCC1, 2, and 3a are robustly expressed in cultures of primary astrocytes as determined via rtPCR (Ringel and Plesnila, 2008). In D54-MF glioma cells, KCC is thought to mediate 30-40% of the osmolyte loss, as determined using the KCC-specific inhibitor DIOA in a manner that took advantage of the temperature-sensitivity of KCC compared to the relatively temperature-independent channel-mediated flux (Ernest et al., 2005).

Swelling-activated Cl^- channels

While a portion of the Cl^- efflux that is observed during regulatory volume decrease is mediated by the K^+/Cl^- symporter (as discussed above), the majority of Cl^- efflux occurs via a volume-sensitive Cl^- channel(s). Swelling-activated Cl^- channels display very similar electrophysiological, biophysical, and pharmacological properties in both neural and non-neural cell types. These channels, which exhibit a conductance of 40-78 pS, are typically outward rectifiers that have inactivating kinetics at positive potentials. Permeability characteristics of swelling-activated Cl^- channels ($I^- > NO_3^- > Cl^- > F^-$) correspond to Eisenman's anion permeability sequence (Nilius and Droogmans, 2003) and require the presence of ATP, but not ATP hydrolysis, since ATP can be replaced by non-hydrolyzable analogues of ATP, ADP, or GTP (Jackson et al., 1994). Volume-sensitive Cl^- efflux is blocked by the estrogen antagonist tamoxifen as well as numerous broad spectrum anion channel blockers, such as DIDS, NPPB, and DDF. Recently, the ethacrynic acid derivative DCPIB, has been demonstrated to inhibit swelling-activated Cl^- fluxes without effect on other Cl^- channels (Decher et al., 2001). Channels that mediate volume-sensitive Cl^- fluxes are thought to be partially active under basal conditions and are thought to participate in mechanisms regulating: cellular proliferation, apoptotic volume decrease, membrane potential, and glial-neuronal communication (Nilius and Droogmans, 2003; Mulligan and MacVicar, 2006).

Despite extensive characterization of this class of channels, and the recent cloning of some Cl^- channels that are osmosensitive, the identity of the swelling-

activated channel(s) that mediate Cl^- efflux during RVD remains elusive. Potential roadblocks to unveiling the identity of this channel include its ubiquity, which greatly hampers cloning, as well as the absence of a high-affinity ligand (Okada, 2006). Several candidate Cl^- channels have been proposed including members of the chloride channel (ClC) family (particularly ClC-2 and ClC-3), phospholemman, P-glycoprotein, phospholemman, $(p)I_{ClN}$, and the band 3 anion exchanger. Although these proteins are no longer considered to be the ubiquitous channel mediating swelling-activated Cl^- flux, it is believed that they may act to regulate such a channel (Nilius et al., 1997). The possibility that a universally expressed swelling-activated Cl^- channel does not exist and that different channels mediate Cl^- fluxes with similar biophysical and electrophysiological characteristics in different cells cannot be discounted.

Although the existence of Ca^{2+}-stimulated Cl^- channels has long been recognized, the exact identities of these channels have remained elusive until recently. Interestingly, cloning of these channels was successful, in part, because salamander (*Ambystoma mexicanum*) eggs were used in place of traditionally favored frog (*Xenopus laevis*) eggs, which endogenously express a Ca^{2+}-sensitive Cl^- current (Galietta, 2009). TMEM16 is one family of recently cloned Ca^{2+}-sensitive Cl^- channels which includes 10 different proteins with numerous splice variants. Genetic deletion of one of these proteins (TMEM16A) inhibits the volume-sensitive Cl^- flux observed in colonic epithelial cells as well as salivary acinar cells (Almaca et al., 2009). Although the tissue-specific expression of this channel and its Ca^{2+}-sensitivity exclude it from consideration as the channel responsible for the swelling-

activated Cl^- flux in all cells, it is possible that this channel mediates a portion of such a Cl^- current in certain cells, although the possibility that it regulates a ubiquitous channel cannot be excluded. Bestrophin-1, another recently discovered Ca^{2+}-stimulated chloride channel, has recently been found to be highly expressed in mouse astrocytes (Park et al., 2009). The drosophila homologue of bestrophin-1 had been shown to be volume-sensitive (Chien and Hartzell, 2007) giving rise to the possibility that this channel may mediate, in part, murine astrocytic swelling-activated Cl^- efflux, although the volume-sensitivity of astrocytic bestrophin-1 has yet to be reported.

Swelling-activated organic osmolyte release

It has been hypothesized that the swelling-activated efflux of numerous organic osmolytes may share a common efflux pathway. Organic osmolyte release has been observed to be relatively insensitive to both temperature and NaCl concentrations (Pasantes-Morales et al., 1990) and appears to be diffusion-mediated in that the bulk movement of an osmolyte is down its concentration gradient (Sanchez-Olea et al., 1991; Roy and Malo, 1992). Although under physiological conditions this will inevitably lead to a net release of osmolyte (due to the enrichment of intracelluar taurine), under experimental conditions in which unidirectional influx is measured, diffusional taurine influx in linearly increased as the extracellular organic osmolyte concentration is raised during hyposmotic swelling (Schousboe et al., 1991). These characteristics all support the hypothesis that

volume-sensitive organic osmolyte release is channel- (and not transporter-) mediated.

It has been suggested that the volume-sensitive organic osmolyte flux is mediated by the swelling-activated Cl^- channel. Although this Cl^- channel has not yet been molecularly identified (as discussed above), volume-sensitive Cl^- release does share a remarkably similar pharmacological profile with volume-sensitive organic osmolyte efflux with regards to broad spectrum anion channel inhibitors such as NPPB and DDF (Sanchez-Olea et al., 1996; Nilius et al., 1997; Kirk and Strange, 1998; Junankar and Kirk, 2000; Abdullaev et al., 2006). Both swelling-activated Cl^- and organic osmolyte fluxes in primary cultures of astrocytes and SH-SY5Y neuroblastoma are also inhibited by DCPIB, a selective inhibitor of volume-sensitive Cl^- channel activity (Abdullaev et al., 2006; Cheema et al., 2007; Ramos-Mandujano et al., 2007). Additional evidence in support of a shared release pathway for Cl^- and organic osmolytes comes from electrophysiological studies that have demonstrated that volume-activated Cl^- channels are permeable to taurine, glutamate, and aspartate when these amino acids are in their anionic form (Banderali and Roy, 1992; Jackson and Strange, 1993; Boese et al., 1996). Collectively, these observations suggest that both swelling-activated Cl^- and organic osmolyte fluxes share a common putative channel which has been referred to as the 'volume-sensitive organic osmolyte and anion channel' (VSOAC), or alternatively the 'volume-regulated anion channel' (VRAC).

In contrast, marked differences with regards to the pharmacological inhibitor profile for organic osmolyte and Cl^- efflux have been observed for some non-neural

cells (Lambert and Hoffmann, 1994; Sanchez-Olea et al., 1995). Furthermore, in HeLa cells, the kinetics of activation and inactivation of taurine and Cl^- efflux differ considerably, a result inconsistent with a common pathway for organic osmolytes and Cl^- (Stutzin et al., 1999). The existence of a common volume-sensitive efflux pathway that is shared amongst organic osmolytes has also been called into question due to the selective activation / inhibition of specific organic osmolytes in some cells. It has been shown that hyposmolarity induces taurine efflux without stimulating the release of other organic osmolytes in both pituicytes and CHP-100 neuroblastoma cells (Basavappa et al., 1996; Miyata et al., 1997). Furthermore, application of NPPB on hippocampal slices or tyrosine kinase inhibitors on primary astrocytes has been demonstrated to inhibit swelling-activated taurine, but not aspartate, efflux (Mongin et al., 1999b; Franco et al., 2001).

In summary, organic osmolytes are released during hypotonic swelling via pathways that are predominantly thought to be channel-mediated. There is a large amount of evidence that supports the hypothesis that organic osmolytes, as a class, share a similar efflux pathway and may exit via a shared channel. In addition, there is substantial pharmacological and electrophysiological evidence to suggest that both organic osmolyte and Cl^- fluxes are mediated via a common channel (VSOAC). However, inconsistencies in the regulation of various osmolyte fluxes as well as variations in their pharmacological profile have raised the possibility that different organic osmolytes may exit via disparate mechanisms and that these mechanisms may be distinct from those that mediate Cl^- release.

Cell volume sensing mechanisms

In order for cells to regulate their volume they must be able to sense changes in cell volume and consequently initiate signaling pathways to activated RVD. Furthermore, these sensors must be able to halt volume regulatory processes once cell volume is returned to normal. To date the mechanism(s) whereby cell swelling is initially sensed by putative 'osmotic sensors' remains an enigma. Current osmosensing candidates include (i) modulation of mechano-sensitive transmembrane proteins (ii) changes in protein function due to changes in macromolecular crowding / ionic strength, and (iii) cell swelling-mediated cytoskeletal rearrangements.

Several volume-sensitive channels have been identified that could participate in volume sensing, such as the two pore potassium channels TREK-1 and TRAAK (Hoffmann et al., 2009), and Ca^{2+}-permeable TRP channels (Pedersen and Nilius, 2007). In addition, integrins and tyrosine kinases (TKs) have been demonstrated to exhibit increased activity during hypotonicity (Pasantes-Morales et al., 2006a). There is general agreement that TK activity is required for the activation of VSOAC in neural tissues based upon the ability of TK inhibitors such as genistein or tyrphostins to attenuate Cl^- and organic osmolytes efflux, and the ability of tyrosine phosphatases to potentiate such fluxes (Sinning et al., 1997; Crepel et al., 1998; Mongin et al., 1999a; Deleuze et al., 2000; Morales-Mulia et al., 2001; Heacock et al., 2004; Cohen, 2005). However it is not clear whether TKs are the originator of

volume-sensitive signal transduction pathways or are simply a down-stream effector of the volume sensor.

With regards to mechano-sensitive-mediated volume sensing there is controversy as to the extent that cell swelling will alter the biophysical properties of the membrane. Because the plasma membrane is not perfectly spherical and is highly folded it is believed that swelling-mediated changes in membrane stretch/tension would be small and transient in nature (Koivusalo et al., 2009), thus raising skepticism as to the import of such mechanisms to volume sensing. Changes in macromolecular crowding that occur as a result of dilution of the intracellular milieu during cell swelling have also been proposed to initiate volume-regulatory processes. Whereas changes in macromolecular crowding / ionic strength have been demonstrated to alter protein function (Lang et al., 1998a) to date no such proteins have been convincingly demonstrated to initiate cell volume regulatory processes.

Another hypothesized cell volume sensor involves cytoskeletal integrity. Rearrangement of the actin cytoskeleton has been observed to occur in certain cell types in response to hypotonicity via a mechanism that, at least in some cases, is mediated by the small molecular weight GTPase Rho (Vazquez-Juarez et al., 2008b). Furthermore, disruption of the cytoskeleton has been demonstrated to preclude the ability of certain cells to undergo RVD (Papakonstanti and Stournaras, 2007). However, cytoskeletal rearrangement upon cell swelling is not universal and in neural cell types disruption of the cytoskeleton has no effect on their ability to perform RVD (Kimelberg et al., 1990; Heacock et al., 2006a). Although none of

these candidate volume sensors can fully explain how cell volume regulatory processes are initiated it may be that a combination of such mechanisms may collectively trigger RVD in a tissue-specific manner.

Regulation of osmolyte efflux by G-protein-coupled receptors

Regulation of cell volume has long been believed to be an intrinsic property of cells. However, evidence is accumulating to suggest that volume-dependent osmolyte efflux can be significantly enhanced following the activation of specific cell-surface G-protein-coupled receptors (GPCRs) (Fig. 1.4). Receptor regulation of osmolyte release, and accordingly cell volume regulation, has been documented to occur in both neural and non-neural cells (for review, see Fisher et al., 2008). The ability of GPCRs to contribute to volume regulatory mechanisms is of particular significance in neural cells given the widespread occurrence of these receptors in the CNS and the critical need to regulate the volume of brain cells within narrow limits. Furthermore, because many of the organic osmolytes released are neuroactive, the contribution of the efflux process to both physiological and pathophysiological events needs to be considered.

The first indication that receptor activation may play a role in osmoregulation was obtained by Bender et al. (1993), who noted that when cultured astrocytes were exposed to hypotonic media, the ensuing volume correction could be accelerated by inclusion of specific ligands known to activate GPCRs, e.g. endothelin or norepinephrine. Indirect evidence for the potential involvement of GPCRs in

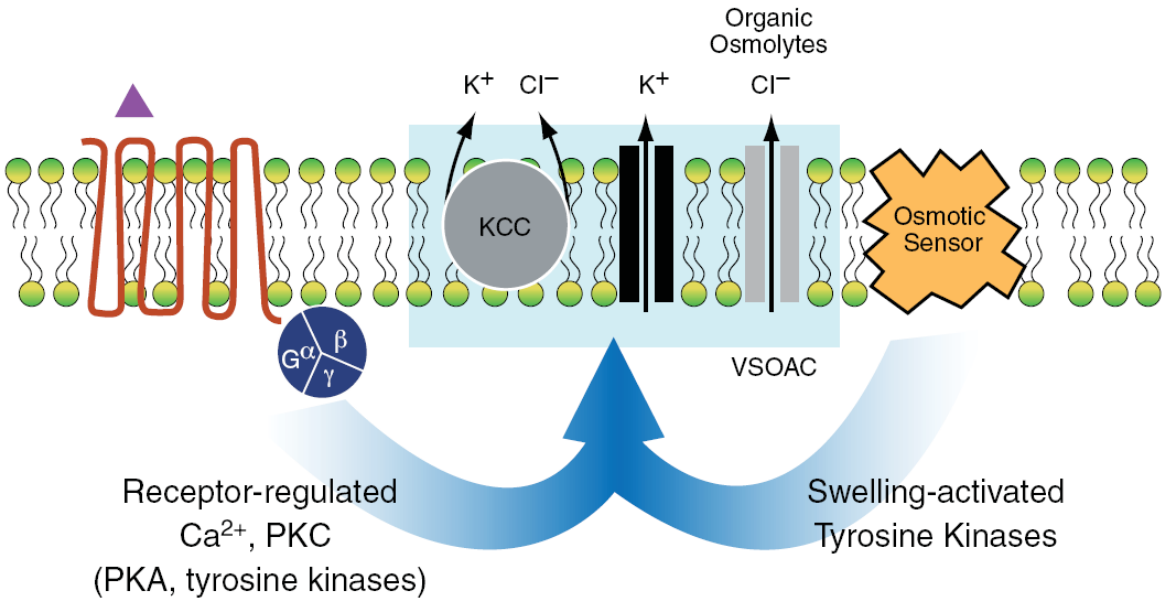

Figure 1.4 Diagrammatic representation of swelling-activated (basal)- and G-protein-coupled receptor (GPCR)-mediated osmolyte release from neural cells. A reduction in osmolarity is proposed to trigger a hypothetical osmotic sensor which results in tyrosine kinase (TK)-dependent activation of the volume-sensitive organic osmolyte and anion channel (VSOAC), K^+ channels and the KCC transporter, with the attendant efflux of K^+, Cl^- and organic osmolytes. The release of these osmolytes can also be enhanced under hyposmotic conditions by the activation of certain GPCRs (shown in red) following the addition of agonist (purple triangle). Receptor-mediated osmolyte release is often demonstrated to be dependent upon Ca^{2+} and protein kinase C (PKC) activity. The involvement of protein kinase A (PKA) and TKs in GPCR regulation of osmolyte efflux is less frequently documented. Although basal and GPCR-mediated osmolyte fluxes are activated by distinct mechanisms the same (or similar) channels and transporters are utilized (Fisher et al., 2008).

osmolyte release was also obtained from experiments in which increases in $[Ca^{2+}]_i$, protein kinase C (PKC), or protein kinase A (PKA) activity were observed to potentiate the volume-dependent efflux of osmolytes from astrocytes, C6 glioma or differentiated NT2-N neurons (Strange et al., 1993; Novak et al., 2000; Moran et al., 2001). However, only in the last few years has the potential contribution of GPCRs to the regulation of osmolyte efflux from neural cells been systematically evaluated and mechanistic aspects examined.

Regulation of osmolyte release has been observed in response to a variety of distinct classes of neurotransmitters or neuromodulators including neuropeptides (vasopressin, endothelin or oxytocin), cholinergic or adrenergic agonists, adenosine, ATP, glutamate, thrombin or lysophospholipids (Table 1). The concentrations of the neurotransmitters / neuromodulators required to elicit osmolyte release *in vitro* (nanomolar to micromolar) are well within the range of those found in the CNS (McIlwain and Bachelard, 1971; Das and Hajra, 1989; Edsall and Spiegel, 1999; Toman and Spiegel, 2002; Hua et al., 2003a; Hua et al., 2003b) and the receptor subtypes identified to regulate osmolyte efflux can also be found within the CNS. Although a diverse range of GPCRs has been implicated in osmoregulation in neural preparations, a number of common signaling characteristics are apparent. Many (but not all) of the receptor subtypes identified to increase volume-dependent osmolyte efflux are also those reported to couple to Ca^{2+} homeostasis and PKC activation, e.g. P_{2Y}, M3 muscarinic cholinergic receptors (mAChRs), protease-activated receptor 1 (PAR-1), and V_{1A} (Mongin and Kimelberg, 2002; Heacock et al., 2004; Rosso et al., 2004; Cheema et al., 2005; Cheema et al., 2007).

Table 1.1 Receptor-mediated osmoregulation in neural cells.

Receptor	Subtype	Signaling Pathways	Cell Type	Parameter	Reference
Adenosine	A_1	PKA	Retina	Cell Swelling	Uckermann et al., 2006
	A_1	PKA / PI3K / PKB	Retina	Cell Swelling	Wurm et al., 2008
	A_{2B}	cAMP/PKA	Pituicytes	Taurine Flux	Pierson et al., 2007
Purinergic	P_{2Y}	ND	Retina	Cell Swelling	Wurm et al., 2008
	P_{2Y}	Ca^{2+} / PKC / CaM / CaMKII	Cortical astrocytes	D-Asp Flux	Mongin and Kimelberg, 2002, 2005
	P_{2Y}	ND	Cortical astrocytes	D-Asp/Cl^- Flux	Abdullaev et al., 2006
	ND	Ca^{2+}	Pituicytes	Taurine Flux	Rosso et al., 2004
	ND	ND	Hippocampal neurons	Taurine/Cl^- Flux	Li and Olson, 2004
	ND	ND	Substantia nigra	Taurine Flux	Morales et al., 2007
Bradykinin	ND	Ca^{2+}	Pituicytes	Taurine Flux	Rosso et al., 2004
Endothelin	ND	PI Hydrolysis	Cortical astrocytes	Cell Swelling	Bender et al., 1993
Erythropoietin	ND	Jak-2 / ERK1/2	Retina	Cell Swelling	Krugel et al., 2010
Glutamate	AMPA	ND	Substantia nigra	Taurine Flux	Morales et al., 2007
	mGluR	ND	Retina	Cell Swelling	Wurm et al., 2008
LPA	ND	Ca^{2+} / PKC	SH-SY5Y	Taurine Flux	Heacock et al., 2006a
	ND	ND	SH-SY5Y	K^+ Flux	Foster et al., 2008
NE	ND	PI Hydrolysis	Cortical astrocytes	Cell Swelling	Bender et al., 1993
	β-AR	PKA	Cortical astrocytes	Taurine Flux	Moran et al., 2001
mAChR	M3	PKC / Ca^{2+} / Tyr kinases	SH-SY5Y	D-Asp/Taurine Flux	Heacock et al., 2004
	M3	Ca^{2+}/PKC	SH-SY5Y	Inositol Flux	Loveday et al., 2003
	M3	Ca^{2+}/PKC	SH-SY5Y	Cl^- Flux	Cheema et al., 2007
	M3	Ca^{2+}/PKC	SH-SY5Y	K^+ Flux	Foster et al., 2008
S1P	ND	Ca^{2+} / PKC	SH-SY5Y	Taurine Flux	Heacock et al., 2006a
	ND	ND	SH-SY5Y	K^+ Flux	Foster et al., 2008
PAR	PAR-1	ND	Cortical astrocytes	Taurine Flux	Cheema et al., 2005
	PAR-1	Ca^{2+} / PKC / CaM / PI3K	Cortical astrocytes	D-Asp Flux	Ramos-Mandujano et al., 2007
	ND	PI Hydrolysis	Cortical astrocytes	Cell Swelling	Bender et al., 1993
	PAR-1	Ca^{2+} / PKC	SH-SY5Y	Taurine Flux	Cheema et al., 2007
	PAR-1	ND	SH-SY5Y	Cl^- Flux	Cheema et al., 2007
	PAR-1	ND	SH-SY5Y	K^+ Flux	Foster et al., 2008
	PAR-1	Ca^{2+} / PKC	1321N1 astrocytoma	Taurine Flux	Cheema et al., 2005
	PAR-1	Ca^{2+} / Rho GTPase	1321N1 astrocytoma	ATP Flux	Blum et al. 2010
Vasopressin	V_{1A}	Ca^{2+}	Pituicytes	Taurine Flux	Rosso et al., 2004
	V_{1A}	ND	Neocortex	Cell Swelling	Niermann et al., 2001
VEGF	VEGFR-2	Ca^{2+} / PLC / PKC / *src*	Retina	Cell Swelling	Wurm et al., 2008

In the cell types where it has been examined, activation of GPCRs promotes the efflux of both inorganic and organic osmolytes (Abdullaev et al., 2006; Cheema et al., 2007; Foster et al., 2008) via mechanisms that are inhibited by VSOACinhibitors NPPB, DDF, and DCPIB (Abdullaev et al., 2006; Cheema et al., 2007; Ramos-Mandujano et al., 2007). These results suggest that GPCR-mediated osmolyte fluxes activate the same (or similar) channels and transporters as those activated by hyposmolarity in the absence of agonist (swelling-activated or basal efflux).

GPCR activation has also been demonstrated to lower the threshold osmolarity (set-point) at which osmolyte efflux occurs. This was first observed by Mongin and Kimelberg (2002) who demonstrated that limited reductions in osmolarity of the magnitude that might be encountered *in vivo*, i.e. 5-10%, elicited little or no increase in the release of D-aspartate from cortical astrocytes. However, inclusion of ATP, which activates P_{2Y} receptors, resulted in a 5-10 fold increase in aspartate release under these conditions of limited hyposmolarity. Additional evidence that GPCR activation can alter the set-point for osmolyte release has been obtained for SH-SY5Y neuroblastoma cells (Heacock et al., 2004). Although these cells release taurine in response to hypotonicity, a statistically significant increase over that observed under isotonic conditions is not achieved until the osmolarity of the medium has been reduced by >30% (Fig. 1.5b). In contrast, in the presence of a muscarinic cholinergic agonist, the efflux of taurine is significantly increased over basal even under isotonic conditions and a further enhancement of release occurs under conditions of limited reductions in osmolarity (6-16%: Fig. 1.5a). Thus, in SH-

SY5Y cells activation of mAChRs lowers the osmotic threshold for taurine release from -34% to 0-5%. mAChR-mediated taurine efflux is attenuated under hyperosmotic conditions (Fig. 1.5b) a result that is consistent with thrombin-stimulated taurine release in SH-SY5Y cells and ATP-stimulated aspartate release in cortical astrocytes (Mongin and Kimelberg, 2002; Cheema et al., 2007). mAChR activation also reduces the osmotic set-point for $^{86}Rb^{+}$ efflux (a radioactive K^{+} homologue) from -40% to 0-5% (Fig. 1.5c). This ability of GPCR activation to facilitate the release of osmolytes by lowering the set-point has also been observed in response to activation of PAR-1, sphingosine 1-phosphate (S1P), and lysophosphatidic acid (LPA) receptors in the SH-SY5Y cell line (Heacock et al., 2006a; Cheema et al., 2007) and for PAR-1 receptors in cortical astrocytes and 1321N1 astrocytoma cells (Cheema et al., 2005; Ramos-Mandujano et al., 2007). The ability of GPCRs to both increase the magnitude of osmolyte release, and to lower the set-point for efflux, may provide a physiological mechanism whereby neural cells are able to respond to the small reductions in osmolarity that would be encountered during hyponatremia, a condition in which decreases in plasma osmolarity of > 20% is rarely observed.

Although the majority of studies have been conducted with cultured cells, there is evidence that receptor-mediated regulation of volume control under hyposmotic conditions can also be demonstrated in more integrated preparations. Thus, activation of A_1 adenosine, erythropoietin, P_{2Y}, mGluR, and VEGF receptors has been linked to an inhibition of osmotic glia swelling in the intact retina (Krugel et al., 2010; Uckermann et al., 2006; Wurm et al., 2008). Furthermore, evidence has

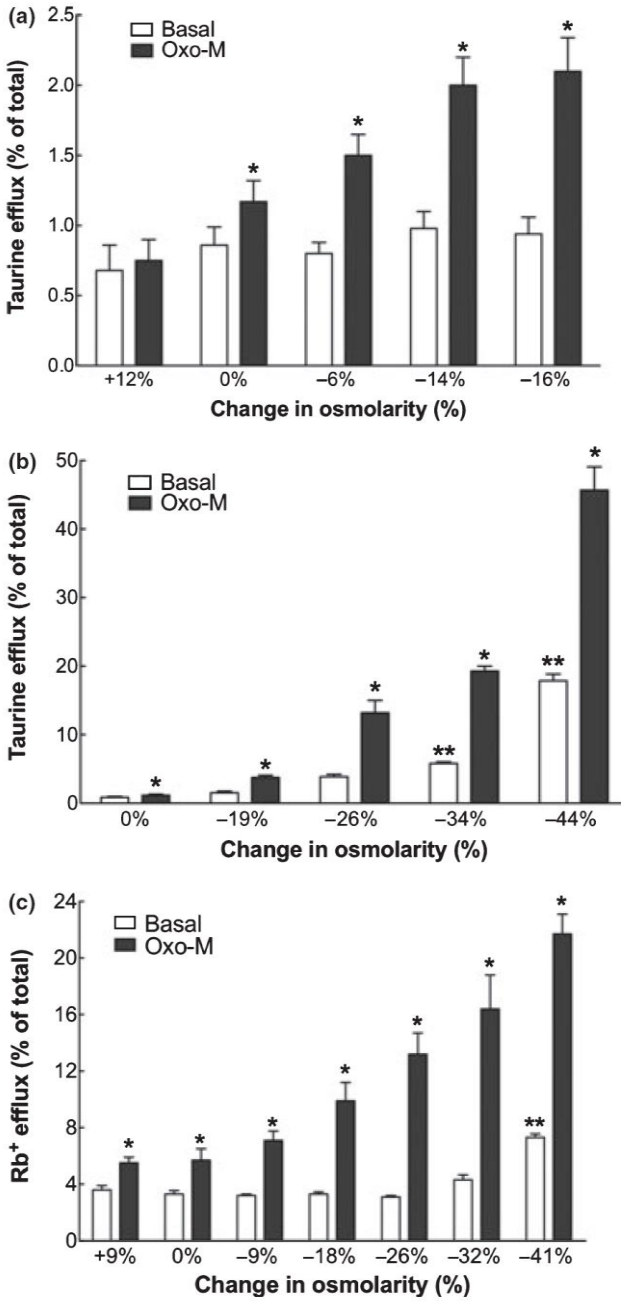

Figure 1.5 Basal- and oxotremorine-M (Oxo-M)-stimulated efflux of taurine and the K^+ radiotracer $^{86}Rb^+$ as a function of osmolarity in SH-SY5Y neuroblastoma cells. SH-SY5Y cells pre-labeled with [^{14}C]taurine or $^{86}Rb^+$ were incubated in buffers of varying osmolarity for either 5 min ($^{86}Rb^+$) or 20 min (^{14}C taurine) with or without the muscarinic cholinergic agonist Oxo-M (100 μM). (a) taurine efflux under relatively modest changes in osmolarity. (b) taurine efflux under conditions of pronounced changes in osmolarity. (c) $^{86}Rb^+$ efflux over a range of osmolarities. Adapted from (Fisher et al., 2008).

been obtained for the involvement of both glutamatergic (AMPA / kainite) and purinergic receptors in the regulation of taurine release from osmotically stressed substantia nigra (Morales et al., 2007).

Further support for the physiological significance of GPCR-mediated cell volume regulation has come from the observation that hypotonicity stimulates the release of endogenous ligands that activate these receptors such as ATP, glutamate, norepinephrine, acetylcholine, and endothelin. Hyposmolarity has been reported to induce ATP release from multiple neural cells in culture via exocytotic (both Ca^{2+}-sensitive and Ca^{2+}-insensitive), channel-mediated (VSOAC), and transporter-mediated mechanisms (Franco et al., 2008). In addition, increases in glutamate, GABA, and norepinephrine release have been observed in cortical synaptosomes subjected to hyposmotic stress. In these preparations, osmotically-sensitive glutamate and GABA release has been demonstrated to occur via multiple pathways (Tuz et al., 2004), whereas norepinephrine release has been observed to occur primarily via Ca^{2+} and PKC-dependent exocytosis both in cortical synaptosomes (Tuz and Pasantes-Morales, 2005), and chromaffin cells (Moser et al., 1995; Amatore et al., 2007). In support of the above studies performed in cell culture, hyposmotically-stimulated ATP and glutamate release has also been observed *in vivo* in rat cortex and hippocampal slices (Franco et al., 2001; Phillis and O'Regan, 2002; Phillis, 2004). Osmosensitive-neurotransmitter release is not unique to neuronal cells as hyposmotic or mechanical perturbations have been demonstrated to stimulate the efflux of both glutamate and endothelin-1 from astrocytes (Ostrow et al., 2000; Liu et al., 2006; Pangrsic et al., 2006). Numerous

ligands including acetylcholine and histamine are also released from various non-neural cells during exposure to hypotonicity (Sernka, 1989; Hanna-Mitchell et al., 2007). Taken collectively, the ability of distinct GPCRs to both promote osmolyte efflux, and lower the set-point for osmolyte release, supports the possibility that hyposmotically-stimulated (via enhanced ligand release) and / or tonic GPCR activation may facilitate volume regulation in neural tissues.

Conversely, GPCR-mediated osmolyte release could potentially have several deleterious consequences as a result of stimulating the release of neuroactive osmolytes (i.e. glutamate). For example, ATP-mediated activation of P_{2Y} receptors significantly increases the ability of cortical astrocytes to release glutamate in response to hyposmolarity (Mongin and Kimelberg, 2002; Mongin and Kimelberg, 2005; Takano et al., 2005). When swollen, astrocytes will release ATP which can then act in a paracrine fashion to promote glutamate efflux (Darby et al., 2003). Paradoxically, as activation of metabotropic glutamate receptors on glial cells is reported to result in cell swelling (Hansson, 1994), released glutamate may actually perpetuate cell swelling. Additionally, although efficient inactivation mechanisms exist for the removal of glutamate from the extracellular space, even modest increases in glutamate can alter neuronal synaptic transmission and impact nearby glia. Glutamate released upon cell swelling is reported to contribute to the slow wave of astrocyte and neuronal depolarization that leads to synaptic depression (Basarsky et al., 1999), and sustained increases in glutamate can also lead to excitotoxicity-mediated neuronal death. Thus, GPCR-activated osmolyte fluxes may

act as a double-edged sword in that they promote the release of potentially harmful glutamate but also beneficially promote brain cell volume regulation.

GPCR-stimulated osmolyte efflux; Signal transduction mechanisms

The signal transduction events that intervene between activation of the 'osmotic sensor' and volume correction under swelling-activated conditions are yet to be defined, and although several candidates have been suggested (Ca^{2+}, PKC, phospholipase A_2, or low molecular weight GTP-binding proteins), only tyrosine kinase (TK) activity appears to be a consistently observed attribute of volume-sensitive osmolyte release (Pasantes-Morales et al., 2006a). There is also a paucity of information regarding the identity of intracellular signaling pathways that mediate GPCR-regulated osmolyte release from neural tissues. Moreover, the information currently available is largely restricted to the release of organic osmolytes. Notwithstanding these considerations, certain features of receptor-stimulated osmolyte release are apparent, most notably a marked dependence on the availability of Ca^{2+}, an observation consistent with the known ability of many of the same receptors to increase $[Ca^{2+}]_i$. In the neural cell types examined thus far (astrocytes and neurotumor cells), depletion of intracellular Ca^{2+} either with thapsigargin or BAPTA-AM significantly attenuates the receptor-mediated release of taurine, glutamate, and myo-inositol (Loveday et al., 2003; Cheema et al., 2005; Mongin and Kimelberg, 2005; Heacock et al., 2006a; Ramos-Mandujano et al., 2007). However, no simple relationship exists between the magnitude of receptor-

mediated increases in $[Ca^{2+}]_i$ and the extent of osmolyte release. For example, the rank order of efficacy for Ca^{2+} mobilization in SH-SY5Y neuroblastoma cells, oxotremorine-M (Oxo-M) > LPA > S1P, differs considerably from that for osmolyte release, S1P > Oxo-M > LPA. In the same cells, down-regulation of inositol trisphosphate receptors that results from prolonged exposure to Oxo-M results in a 50-70% reduction in the ability of S1P and LPA to increase $[Ca^{2+}]_i$ but has no effect on the efficacy with which these two ligands can enhance osmosensitive taurine release (Heacock et al., 2006a). Furthermore, although the rise in $[Ca^{2+}]_i$ that results from the activation of GPCRs reflects both the influx of extracellular Ca^{2+} and mobilization of intracellular Ca^{2+}, a requirement for extracellular Ca^{2+} in osmolyte release is observed only for some receptors (e.g. mAChR in SH-SY5Y cells and PAR-1 receptors in astrocytes: Heacock et al., 2006a; Ramos-Mandujano et al., 2007). Taken collectively, these results suggest that, although Ca^{2+} availability appears to be a prerequisite for maximal receptor-stimulated osmolyte release, both the degree of dependence and source of Ca^{2+} may differ, depending on the cell type and / or receptor involved. One potential down-stream effector for Ca^{2+} is calmodulin, as trifluoperazine, chlorpromazine, or W7 all inhibit ATP- or thrombin-stimulated osmolyte release from astrocytes (Mongin and Kimelberg, 2005; Ramos-Mandujano et al., 2007).

A correlation between receptor-regulated changes in osmoregulation and Ca^{2+} was first noted by Bender et al. (1993), who proposed a link between receptor-mediated increases in phospholipase C (PLC) activity and RVD. However, lysophospholipid receptors that mobilize Ca^{2+} and facilitate osmolyte efflux in SH-

SY5Y neuroblastoma cells do not elicit an increase in the activity of PLC (Heacock et al., 2006a). Furthermore, although PAR-1 receptors enhance osmolyte release from both 1321N1 astrocytoma and SH-SY5Y neuroblastoma cells, the addition of thrombin only increases PLC activity in the former cell line (Cheema et al., 2005; Cheema et al., 2007). Thus, it is now apparent that osmolyte release can be facilitated by Ca^{2+}-mobilizing receptors that operate via PLC-dependent or –independent mechanisms.

An additional prerequisite for maximal receptor-stimulated osmolyte release is that of PKC-activity. For astrocytes and neurotumor cells, pre-incubation of cells with inhibitors of PKC, such as chelerythrine or bisindolylmaleimide, results in an attenuation of receptor-stimulated osmolyte efflux (Cheema et al., 2005; Mongin and Kimelberg, 2005; Heacock et al., 2006a; Ramos-Mandujano et al., 2007). Down-regulation of PKC following overnight exposure of SH-SY5Y cells to PMA also severely attenuates mAChR-stimulated myo-inositol release (Loveday et al., 2003). Recently, evidence for the involvement of two conventional isoforms of PKC, namely α and β1, in ATP-mediated D-aspartate release from astrocytes has been obtained from experiments in which the activity of these enzymes was attenuated by either siRNA knock-down, or the inclusion of cell-permeable inhibitory peptides. Of particular significance was the observation that down-regulation of both PKCα and PKCβ1 was required for inhibition, suggesting a cooperative interaction of these two isoforms (Rudkouskaya et al., 2008). Under conditions in which both PKC is inhibited and intracellular Ca^{2+} depleted, the ability of GPCRs to regulate organic osmolyte efflux is essentially abolished (Mongin and Kimelberg, 2005; Heacock et

al., 2006a). However, in SH-SY5Y cells PAR-1-mediated Cl^- efflux (as measured with $^{125}I^-$) is relatively insensitive to depletion of Ca^{2+} or inhibition of PKC, even though the release of taurine and $^{125}I^-$ occurs via pharmacologically similar membrane channels (Cheema et al., 2007). These results, which are in agreement with those previously obtained for P_{2Y} receptor activation in hepatoma cells (Junankar et al., 2002), suggest that distinct biochemical requirements may exist for the release of inorganic and organic osmolytes. It should be stressed that the swelling-activated release of organic osmolytes, in contrast to that observed under GPCR-stimulated conditions, also appears to be relatively independent of both Ca^{2+} and PKC in neural cells (Loveday et al., 2003; Mongin and Kimelberg, 2005; Takano et al., 2005; Heacock et al., 2006a; Cheema et al., 2007; Rudkouskaya et al., 2008). These observations are consistent with the proposal that distinct mechanisms underlie basal- and receptor-mediated osmolyte efflux (Mongin and Kimelberg, 2005). The targets of receptor-stimulated Ca^{2+}- and PKC-mediated signal transduction pathways remain elusive but could include direct effects on volume-sensitive channels, proteins that modulate the activity / trafficking of such channels, or the 'volume sensor' itself.

A significant body of evidence obtained from non-neural cells suggests that both receptor TKs (such as epidermal growth factor; Franco et al., 2004) and non-receptor TKs may play an important role in GPCR regulation of osmolyte release, with members of the *src* family being the preferred candidate (Vazquez-Juarez et al., 2008b). These observations are consistent with the known 'cross-talk' between GPCR and TK signaling pathways (Luttrell and Luttrell, 2004). However, only a

limited amount of information is available regarding the importance of GPCR / TK interactions in promoting osmolyte efflux in neural cells. ATP-stimulated D-aspartate release from astrocytes is inhibited 50-60% following inclusion of either tyrphostin A51 or PP2, a *src* TK inhibitor. However, this inhibition is only observed during limited reductions in osmolarity (5%) and is absent when osmolarity is further reduced (30%) (Mongin and Kimelberg, 2005). In contrast, in SH-SY5Y cells mAChR-mediated taurine / D-aspartate release is inhibited by 60-70% by inclusion of the tyrphostin AG-18 and enhanced upon tyrosine phosphatase inhibition, even when osmolarity is reduced by 30% (Heacock et al., 2004).

Non-receptor-mediated increases in cAMP have been reported to facilitate osmolyte efflux or swelling-activated Cl^- currents in several tissues (Strange et al., 1993; Shimizu et al., 2000; Moran et al., 2001). In murine Ltk fibroblasts, prostaglandin E_1 stimulates the osmosensitive release of taurine via an EP_2 receptor. This effect was mimicked by the addition of cAMP analogues and attenuated in a mutated Ltk fibroblast cell line (RAB-10) in which the regulatory subunit of PKA exhibited a substantial reduction in its affinity for cAMP (Heacock et al., 2006b). However, norepinephrine-stimulated release of taurine from astrocytes is the only documented example of cyclase-linked receptor-mediated osmolyte release from neural cells (Moran et al., 2001). In summary, it appears likely that in neural cells GPCR regulation occurs via several distinct mechanisms and that Ca^{2+} availability and PKC activity play a major role in this process with ostensible roles for PLC, PKA, and TK activity as well.

Mediators of osmolyte uptake

The majority of studies examining osmolyte uptake have focused on hypertonic conditions, where increased osmolyte uptake is the primary mechanism through which volume regulation occurs (for reviews, see Ibsen and Strange, 1996; Lang et al., 1998a; Fisher et al., 2002; Han et al., 2006). Because the fundamental goal of cell volume regulation under hypotonic conditions is to expel osmolytes, the ability of hypotonicity to alter osmolyte uptake has received less attention. However, the net loss of an osmolyte from a cell is equal to the sum of changes in the efflux and uptake of that osmolyte. Accordingly, changes in both efflux and uptake have the potential to affect the net release of a given osmolyte. Many of the transporters that mediate osmolyte uptake under isotonic conditions are subject to regulation by PKA, PKC, and other signaling pathways that are initiated by GPCRs that activate volume-dependent osmolyte efflux. Although several studies have examined the ability of these GPCRs to regulate osmolyte efflux, their effects on volume-sensitive osmolyte uptake, a potentially key contributor in determining the net flux of an osmolyte, has not been examined.

With regards to characterization of osmolyte uptake under hypotonic conditions, that of K^+ has received the most attention. K^+ uptake in neural cells is mediated in large part by the energy-transducing Na^+/K^+ ATPase which translocates 3 K^+ ions into the cell in exchange for 2 intracellular Na^+ ions and is sensitive to inhibition by ouabain. Members of the SLC12A family of transporters such as the NKCC and KCC also play key roles in K^+ uptake in neural cells and these

transporters are characterized by their sensitivity to loop diuretics such as furosemide and bumetanide. NKCC and KCC mediate the electroneutral symport of $1Na^+:1K^+:2Cl^-$, and $1K^+:1Cl^-$ respectively (Russell, 2000; Gagnon et al., 2007). Hypotonicity has been demonstrated to increase K^+ uptake via both Na^+/K^+ ATPase and NKCC activity in cultures of primary astrocytes and astrocytomas (Mongin et al., 1994; Mongin et al., 1996), and stimulation of NKCC has also been observed in response to cell swelling caused by exposure to elevated extracellular K^+ concentrations (Su et al., 2002).

Several intracellular signaling pathways that are commonly activated by GPCRs known to regulate osmolyte efflux also have been demonstrated to alter K^+ transporter function. Activation of cAMP/PKA, PKC, and Ca^{2+} / CaM by various means including β-adrenergic and D1 dopamine receptor activation has been demonstrated to alter Na^+/K^+ ATPase activity (Zhang et al., 2008). Similarly, NKCC has been demonstrated to possess several PKA and PKC consensus phosphorylation sites (Russell, 2000) and either addition of norepinephrine, or activation of glutamate receptors (mGluR, NMDA), has been demonstrated to increase NKCC mediated uptake in cells under isotonic conditions (Sun and Murali, 1998; Schomberg et al., 2001).

The above evidence suggests that transporters that mediate K^+ influx are activated by cell swelling, a mechanism that may act to maintain intracellular KCl concentrations during hypotonic-swelling induced cell volume regulation. Although these transporters, under isotonic conditions, also appear to be subject to regulation by signaling cascades utilized by GPCRs known to participate in cell volume

regulation, the ability of such GPCRs to alter K^+ and Cl^- uptake (especially under hypotonic conditions) has yet to be examined.

Swelling-mediated effects on the influx of taurine have only recently been examined and potential hypotonic regulation of other organic osmolytes has yet to be assessed. The taurine transporter (TauT) can exist in two different isoforms, both found in the brain (Liu et al., 1992; Smith et al., 1992; Pow et al., 2002), and is a member of the SLCA6 family of transporters and thus is structurally similar to the dopamine, norepinephrine, and serotonin transporters. In contrast to the effects of cell swelling on K^+ transporters, hypotonicity has been demonstrated to reduce the activity of the taurine transporter (TauT) in hippocampal neurons as well as in ascites tumor cells (Hoffmann and Lambert, 1983; Olson and Martinho, 2006). However, taurine uptake was unaffected by hypotonicity in astrocytes and cerebellar granule cells (Sanchez-Olea et al., 1991; Schousboe et al., 1991). Inhibition of taurine uptake could effectively potentiate the net release of taurine, a relatively inert osmolyte, from certain neural cells.

Whereas taurine is considered a relatively inert osmolyte, the organic osmolyte glutamate can induce excitotoxicity when its extracellular concentration is not properly regulated. The extracellular concentration of glutamate is precisely controlled via the activity of excitatory amino acid transporters (EAATs). There are five EAAT isoforms, four of which are found in neural tissues; EAAT1-2 are thought to be primarily found in glia while EAAT3-4 are localized to neurons. Glutamate is taken up into cells along with 3 Na^+ ions and one H^+ with the concurrent counter-transport of 1 K^+ ion (Kanai and Hediger, 2004). While to date no studies have

examined the effect of hypotonicity on EAAT function, it has been demonstrated that conditions of ischemia, hypoxia, or high extracellular potassium reduce glutamate uptake in neural cells (O'Neill et al., 1994; Kimelberg et al., 1995), and in the case of ischemia, cause EAAT-mediated glutamate release (Rossi et al., 2000). Under isotonic conditions EAATs have been demonstrated to have their trafficking / activity altered by a diverse array of kinases including PKC, PKB, PI3K, and MAPK although this regulation is subject to considerable heterogeneity among subtypes (Beart and O'Shea, 2007). TauT is also sensitive to negative regulation by PKC (Han et al., 2006) providing circumstantial evidence that GPCRs that mediate osmolyte efflux may be able to alter TauT and / or EAAT activity under hypotonic conditions, although prior to the current study this had not yet been examined.

Inorganic osmolytes are released acutely from cultured neural cells to the same or greater extent than is observed for organic osmolytes under both basal (swelling-activated) and receptor stimulated conditions (Fig. 1.5, Abdullaev et al., 2006; Cheema et al., 2007). In contrast, chronic hyponatremia results in a disproportionately greater percentage loss of organic osmolytes from the brain (Fig. 1.3, Lien et al., 1991; Videen et al., 1995; Massieu et al., 2004; Abdullaev et al., 2006; Cheema et al., 2007). Furthermore, when astrocytes are cultured under chronic hyposmotic conditions, organic osmolytes, but not K^+, are lost from the cells (Olson, 1999). This creates an enigma in that the acute efflux of both organic and inorganic osmolytes appears to be of a similar magnitude *in vitro*, yet they are differentially retained *in vivo*. Under hypotonic conditions, the net loss of an osmolyte from a cell is equal to the sum of changes in the efflux and uptake of that

osmolyte. Accordingly, changes in both efflux and uptake have the potential to affect the net release of a given osmolyte. One possible explanation for the apparent paradox outlined above is that, while the release of both inorganic and organic osmolytes is similar under hypotonic conditions, the volume-dependent efflux of inorganic osmolytes is accompanied by a compensatory uptake phase, as originally proposed by Mongin et. al. (1994; 1996). Whereas osmolyte uptake has been extensively examined under conditions of hypertonic stress, the effect of hypotonic conditions and / or GPCR-activation on osmolyte uptake has remained largely unexplored.

Thesis Goals

The underlying hypothesis for my thesis is that receptor-regulation of osmolyte uptake is the primary mechanism whereby neural cells selectively regulate the retention of inorganic and organic osmolytes under hypotonic conditions. Such systematic regulation of osmolyte depletion is crucial given the varying physiological consequences that release of different osmolytes can have, although the mechanisms whereby selective osmolyte retention occur have not been previously elucidated. I was particularly interested in determining the mechanisms whereby mAChRs modulate osmolyte uptake (particularly the quantitatively major osmolytes K^+, taurine and glutamate) in SH-SY5Y neuroblastoma cells, because of the defined signaling pathways activated by mAChRs and the homogeneity of SH-SY5Y cells. However, key findings were verified using multiple GPCRs previously implicated in

volume regulation both in SH-SY5Y cells and in cultures of primary astrocytes when possible.

Although both inorganic and organic osmolytes are released from cells exposed to hyposmotic stress to a similar extent *in vitro* they are differentially retained *in vivo* with a preference for retention of inorganic osmolytes such as K^+. Because the net loss of an osmolyte is the sum of both its efflux and concurrent reuptake, I hypothesized that K^+ retention under hypotonic conditions is facilitated by GPCR-mediated stimulation of volume-sensitive K^+ influx, while the comparative depletion of organic osmolytes such as taurine and glutamate is exacerbated by osmotic- and / or GPCR-mediated decreases in organic osmolyte uptake. Furthermore, given the potential deleterious effects of glutamate, and its retention in the hyponatremic brain when compared to taurine, I hypothesized that glutamate uptake would be differentially-regulated from that of taurine so as to maintain low concentrations of extracellular glutamate. In Chapter 2, I demonstrate that both hypotonicity and mAChR activation stimulate K^+ uptake (monitored using $^{86}Rb^+$), primarily via increased activity of the Na^+/K^+ ATPase and NKCC. Furthermore, the magnitude of these increases in K^+ uptake were demonstrated to be of a magnitude such as to counteract receptor-mediated increases in K^+ efflux during physiologically relevant (<15%) reductions in osmolarity, thus allowing retention of intracellular K^+. In Chapter 3, I further characterized the ability of hypotonicity and mAChR activation to attenuate TauT-mediated ^{3}H-taurine uptake and demonstrated that mAChR-mediated effects on taurine uptake and efflux are mediated via distinct signal transduction pathways. Inhibition of taurine uptake with concurrent enhanced

taurine efflux could exacerbate the depletion of intracellular taurine and explain the severity of taurine depletion from chronically hyponatremic rat brains. Lastly, in Chapter 4, I provide evidence that mAChR activation significantly increases glutamate uptake (as assessed with ^{3}H-D-aspartate) into SH-SY5Y cells via increased surface expression of EAAT3, an effect that is disrupted by hypotonicity. Such a mechanism would allow for glutamate retention during small reductions in osmolarity and account for the intermediate retention of glutamate compared to K^{+} and taurine *in vivo*.

Chapter 2

Activation of Muscarinic Cholinergic Receptors on Human SH-SY5Y Neuroblastoma Cells Enhances both the Influx and Efflux of K^+ under Conditions of Hyposmolarity

Summary

The ability of receptor activation to regulate osmosensitive K^+ fluxes (monitored as $^{86}Rb^+$) in SH-SY5Y neuroblastoma and primary rat astrocytes has been examined. Incubation of SH-SY5Y cells in buffers rendered increasingly hypotonic by a reduction in NaCl concentration resulted in an enhanced basal efflux of Rb^+ (threshold of release, 200 mOsM), but had no effect on Rb^+ influx. Addition of the muscarinic cholinergic agonist, oxotremorine-M (Oxo-M), potently enhanced Rb^+ efflux (EC_{50} = 0.45 μM) and increased the threshold of release to 280 mOsM. Oxo-M elicited a similarly potent, but osmolarity-independent, enhancement of Rb^+ influx (EC_{50} = 1.35 μM). However, when incubated under hypotonic conditions in which osmolarity was varied by the addition of sucrose to a fixed concentration of NaCl, basal- and Oxo-M-stimulated Rb^+ influx and efflux were demonstrated to be dependent upon osmolarity. Basal- and Oxo-M-stimulated Rb^+ influx (but not Rb^+ efflux) were inhibited by inclusion of ouabain or furosemide. Both Rb^+ influx and efflux were inhibited by removal of intracellular Ca^{2+} and inhibition of protein kinase

C activity. In addition to Oxo-M, agonists acting at other cell-surface receptors previously implicated in cell volume regulation enhanced Rb^+ efflux and influx under hypotonic conditions in both SH-SY5Y neuroblastoma and primary rat astrocytes. Oxo-M had no effect on cellular K^+ concentration in SH-SY5Y cells under physiologically relevant reductions in osmolarity (0-15%) unless K^+ influx was blocked. Thus although receptor activation enhances the osmosensitive efflux of K^+, it also stimulates K^+ influx and the latter permits retention of K^+ by the cells.

Introduction

Regulation of cell volume is of prime importance to the CNS due to the restricted volume of the skull (Pasantes-Morales et al., 2000a; Pasantes-Morales et al., 2002b). Even modest alterations in brain volume can have profound effects within the CNS as the spatial relationships between neurons, astrocytes and extracellular space are compromised. Brain cells swell either via changes in plasma osmolarity (hypoosmotic swelling) or in intracellular ion and water distribution (isotonic swelling or cellular/cytotoxic edema). The most prevalent cause of hypoosmotic swelling is a condition known as hyponatremia, which is defined as a reduction in serum Na^+ concentration from a normal value of 145 mM to 136 mM or below. Hyponatremia, which may result from congestive heart failure, nephrotic syndrome, hepatic cirrhosis, inappropriate secretion of anti-diuretic hormone or psychotic polydipsia, occurs in 2.5% of hospitalized patients (Lien and Shapiro, 2007). It disproportionately affects the young and the elderly and causes predominantly neurological symptoms such as lethargy, confusion and coma.

Following hypoosmotic stress, cells swell in proportion to the reduction in osmolarity and then normalize their volume in a recovery process known as regulatory volume decrease in which osmolytes (K^+, Cl^- and small organic molecules) are extruded and cell volume is normalized via the exit of obligated water (McManus et al., 1995). Inorganic osmolytes, such as K^+ and Cl^-, constitute the quantitatively major component of the osmolyte pool (60-70%), whereas organic osmolytes such as taurine, glutamate and inositol comprise the remainder

(Pasantes-Morales et al., 2002b). In most (but not all) tissues, the extrusion of Cl^- and organic osmolytes appears to occur via a common, volume-sensitive organic osmolyte and anion channel, which is primarily permeable to Cl^-, but impermeable to cations (Sanchez-Olea et al., 1996; Lang et al., 1998b; Nilius and Droogmans, 2003; Abdullaev et al., 2006). Although less extensively studied, the efflux of K^+ has been reported to occur via a variety of different K^+ channels including those gated by voltage or activated by stretch, swelling or Ca^{2+} (Pasantes-Morales et al., 2006b).

When monitored *in vitro*, the efflux of both inorganic and organic osmolytes is relatively insensitive to hypoosmotic stress, often requiring reductions in osmolarity (>30%) that are not typically encountered *in vivo*. However, recent studies from this and other laboratories have demonstrated that the volume-sensitive efflux of osmolytes from neural tissues can be enhanced following the activation of certain G-protein-coupled receptors (GPCRs), including the P_{2Y} purinergic (Mongin and Kimelberg, 2002; Mongin and Kimelberg, 2005) (Mongin and Kimelberg, 2002, 2005), M3 muscarinic cholinergic (mAChR: Loveday et al., 2003; Heacock et al., 2004), lysophospholipid (Heacock et al., 2006a) and the protease-activated-1 receptors (Cheema et al., 2005; Cheema et al., 2007; Ramos-Mandujano et al., 2007). Receptor activation not only increases the extent of osmolyte release, but also lowers the threshold osmolarity ('set-point') at which osmolytes are released. The latter observation raises the possibility that tonic agonist activation of cell-surface receptors may permit neural cells to respond to more physiologically relevant reductions in osmolarity.

Although inorganic osmolytes are released from cultured neural cells to the same or greater extent than is observed for organic osmolytes under both basal (swelling-activated) and receptor-stimulated conditions (Abdullaev et al., 2006; Cheema et al., 2007), chronic hyponatremia results in a disproportionately greater percentage loss of organic osmolytes than of inorganic osmolytes from the brain (Melton et al., 1987; Lien et al., 1991; Videen et al., 1995; Pasantes-Morales et al., 2002b; Massieu et al., 2004). One potential explanation for this observation is that, under hypoosmotic conditions, the volume-dependent efflux of inorganic osmolytes is accompanied by a compensatory uptake phase, as previously proposed for K^+ (Mongin et al., 1994; Mongin et al., 1996). However, the issue of whether receptor activation can promote the uptake of osmolytes under hypoosmotic conditions has not, to the best of our knowledge, been previously investigated. To address this question, in the present study we have examined the ability of mAChRs (and other GPCRs) to regulate K^+ homeostasis in human SH-SY5Y neuroblastoma cells and primary cultures of astrocytes under conditions of hypoosmotic stress. The results indicate that receptor activation facilitates **both** the efflux and influx of K^+ in an osmosensitive manner. Under conditions of either isotonicity or limited reductions in osmolarity (15%), the efflux of K^+ is effectively countered by an influx of K^+, such that no net loss of cell K^+ occurs. Only under more pronounced reductions in osmolarity (30%) does the rate of K^+ efflux exceed that of influx and result in a net loss of K^+. Thus, receptor activation serves to regulate both the release and uptake of osmolytes.

Materials and Methods

Materials. Rubidium Chloride ($^{86}Rb^{+}$-labeled; 241 MBq/mg) was obtained from PerkinElmer Life and Analytical Sciences (Shelton, CT). 3-*O*-Methyl-D-[1-^{3}H]glucose (148 GBq/mmol) was from GE Healthcare (Piscataway, NJ). Oxotremorine-M, sphingosine 1-phosphate, thrombin, bumetanide, DIOA, ouabain, furosemide, tetraethylammonium chloride, barium chloride dihydrate, atropine and 3-*O*-methyl-D-glucose were purchased from Sigma-Aldrich (St. Louis, MO). Iberiotoxin, charybdotoxin, apamin, glibenclamide and 4-aminopyridine were obtained from Tocris Bioscience (Ellisville, MO). Chelerythrine, thapsigargin and phloretin were obtained from Calbiochem (San Diego, CA). Lysophosphatidic acid was purchased from Avanti (Alabaster, AL). Dulbecco's modified Eagle medium (DMEM) and 50x penicillin/streptomycin were obtained from Invitrogen (Carlsbad, CA). Fetal calf serum was obtained from Cambrex Bio Science (Walkersville, MD). Tissue culture supplies were obtained from Corning Glassworks (Corning, NY), Starstedt (Newton, NC) and BD BioSciences (Franklin Lakes, NJ). Universol was obtained from Valeant Pharmaceuticals (Costa Mesa, CA).

Cell culture conditions. Human SH-SY5Y neuroblastoma cells (passages 70-89) were grown in tissue culture flasks (75 cm^2/250ml) in 20 ml of DMEM supplemented with 10% (v/v) of fetal calf serum with 1% penicillin/streptomycin. The osmolarity of the medium was 330-340 mOsM. Cells were grown at 37^{o}C in a humidified atmosphere containing 10% CO_2. The medium was aspirated and cells detached

from the flask with a trypsin-versene mixture (Cambrex Bio Science, Walkersville, MD). Cells were then resuspended in DMEM/10% fetal calf serum with penicillin/streptomycin and subcultured into 35-mm, six-well culture plates at a density of 250 to 300,000 cells/well for 4 to 5 days. Cells that had reached 70-90% confluence were routinely used.

Preparation of primary cultures of astrocytes. Neonatal cultures of rat astrocytes were prepared from 2-day old rats (Sprague-Dawley) essentially according to the method previously described (Xiang et al., 2006). Cells that had reached 80-90% confluence were routinely used.

Measurement of K^+ efflux. K^+ efflux from SH-SY5Y neuroblastoma cells or primary cultures of rat astrocytes was determined using $^{86}Rb^+$ as a tracer for K^+. In brief, cells were prelabeled overnight to isotopic equilibrium with 19-37 KBq/ml $^{86}Rb^+$ at 37^{o}C. After prelabeling, the cells were washed three times with 2 ml of isotonic buffer A (142 mM NaCl, 5.6 mM KCl, 2.2 mM $CaCl_2$, 3.6 mM $NaHCO_3$, 1 mM $MgCl_2$, 30 mM HEPES, pH 7.4, and 1 mg/ml D-glucose, ~340 mOsM). Cells were then allowed to incubate in 2 ml of buffer A (370-200 mOsM; routinely rendered either hypertonic or hypotonic by an increase or decrease in NaCl concentration, respectively) in the absence or presence of agonists. In some experiments, osmolarities of the buffers were adjusted under conditions of a constant NaCl concentration (79 mM NaCl) by the addition of sucrose. Osmolarities of buffers were monitored by means of an Osmette precision osmometer (PS Precision

Systems, Sudbury, MA). At the times indicated, aliquots of the extracellular medium (1 ml) were removed and radioactivity determined after the addition of 6 ml of Universol scintillation fluid. The reactions were terminated by rapid aspiration of the buffer and cells lysed by the addition of 2 ml of 0.1 M NaOH. The rate of efflux of $^{86}Rb^{+}$ was calculated as a fractional release/min, i.e., the radioactivity released/min into the extracellular medium as a percentage of the total radioactivity present initially in the cells. The latter was calculated as the sum of radioactivity recovered in the extracellular medium and that remaining in the lysate at the end of the assay. For all measurements, radioactivity released at the zero time point was subtracted from the observed value. Throughout the study "basal" release of $^{86}Rb^{+}$ is defined as that which occurs at a specified osmolarity in the absence of agonists.

Measurement of K^{+} influx. K^{+} influx was determined using $^{86}Rb^{+}$ as a tracer for K^{+}. SH-SY5Y neuroblastoma cells or primary cultures of rat astrocytes were washed twice with 2 ml of isotonic buffer A (~340 mOsM) and then incubated in buffer A (370-200 mOsM routinely rendered either hypertonic or hypotonic, unless otherwise stated, by an increase or decrease in NaCl concentration, respectively) containing $^{86}Rb^{+}$ (28-56 KBq/ml) with or without agonist at 37^{o}C. In some experiments, osmolarities of the buffers were adjusted under conditions of a constant NaCl concentration (79 mM NaCl) by the addition of sucrose. At the times indicated, the extracellular medium was aspirated, cells washed three times with 2 ml of isotonic buffer A and then the cells were lysed with 2 ml of 0.1 M NaOH. Aliquots of lysate (1 ml) were removed and radioactivity determined after the addition of 6 ml Universol

scintillation fluid. In all measurements, radioactivity accumulated at the zero time point was subtracted from the observed value. Protein contents of cell lysates were determined using a bicinchoninic acid protein assay reagent kit (Pierce, Rockford, IL). From the measurement of $^{86}Rb^{+}$ uptake, K^{+} influx was calculated as nmol/mg protein/min with the assumption that $^{86}Rb^{+}$ transport into the cells reflects that of K^{+}.

Intracellular water space. The intracellular water space was measured essentially as previously described (Novak et al., 1999). SH-SY5Y neuroblastoma cells were washed with 5 x 2 ml of buffer A without D-glucose and then incubated in buffer A with increasing extracellular concentrations of 3-*O*-[^{3}H]-methyl-D-glucose at 37^{o}C until equilibrium had been achieved (50 min). Cells were then washed with 5 x 2 ml of ice-cold buffer A without glucose containing 0.1 mM phloretin and lysed with 2 ml of 0.1 M NaOH. Aliquots (1 ml) of lysate were removed and radioactivity determined after the addition of 6 ml Universol scintillation fluid. Intracellular concentrations of 3-*O*-[^{3}H]-methyl-D-glucose were monitored at equilibrium and a plot of this parameter vs. the concentration of extracellular 3-*O*-[^{3}H]-methyl-D-glucose yields a line whose slope is the volume of intracellular water with respect to protein. Determination of water space by this method requires that 3-*O*-methyl-D-glucose not be metabolized or actively transported, and these assumptions were validated by the linearity of the plot and its extrapolation through the origin (Kletzien et al., 1975).

K^{+} mass measurements. SH-SY5Y cells were washed with 2 x 2 ml of isotonic buffer A. Cells were then incubated for 10 min in buffer A (340-230 mOsM rendered

hypotonic by a reduction in NaCl concentration) at 37^{o}C. The extracellular medium was then aspirated, cells were then washed with 2 ml of K^+ free buffer A (142 mM NaCl, 2.2 mM $CaCl_2$, 3.6 mM $NaHCO_3$, 1 mM $MgCl_2$, 30 mM HEPES, pH 7.4, and 1 mg/ml D-glucose, ~335 mOsM) and lysed in 2 ml of 0.1 M NaOH. Protein contents of cell lysates were determined using a bicinchoninic acid protein assay reagent kit (Pierce, Rockford, IL). Lysates from three separate 35 mm wells were combined (total volume 6 ml) and centrifuged at 3,000 x g for 30 min at 5^{o} C. Supernatants were then adjusted to a final pH of between 5 and 11 with 4 N HCl. K^+ values were obtained using a glass combination K^+ electrode (Cole Parmer) and an Acorn Series Ion 6 meter (Oakton Instruments, Vernon Hills, IL).

Data Analysis. All experiments shown were performed in duplicate or triplicate and repeated at least three times. Values quoted are given as means $\pm$ S.E.M. for the number (n) of independent experiments indicated. A two-tailed Student's t test (paired or unpaired) was used to evaluate differences between two experimental groups (level of significance, $p < 0.05$). Ordinary or repeated measures analysis of variance (ANOVA) followed by Dunnett's multiple comparisons test was used for statistical significance of differences between multiple groups. EC_{50} values were obtained using Prism 4.0a (GraphPad Software Inc., San Diego, CA).

Results

Agonist activation of mAChRs on SH-SY5Y neuroblastoma cells enhances both $^{86}Rb^{+}$ influx and $^{86}Rb^{+}$ efflux. When SH-SY5Y cells were exposed to hypotonic buffer A (230 mOsM; ~30% reduction in osmolarity), conditions previously determined to be optimal for the release of organic osmolytes, there was a time-dependent increase in both $^{86}Rb^{+}$ influx and $^{86}Rb^{+}$ efflux under basal conditions ('basal' is defined as influx or efflux monitored at a specified osmolarity in the absence of agonist). Inclusion of Oxo-M (100 μM) elicited a marked enhancement of $^{86}Rb^{+}$ influx over basal at 3 min and thereafter and resulted in a doubling of the rate of $^{86}Rb^{+}$ uptake (Fig. 2.1A). Inclusion of Oxo-M also significantly enhanced the efflux of $^{86}Rb^{+}$ in an approximately linear manner up to 10 min of incubation (rate constants for $^{86}Rb^{+}$ efflux under basal- and Oxo-M-stimulated conditions were 0.85 and 2.61% per min, respectively; Fig. 2.1B). In subsequent experiments, both basal- and agonist-stimulated $^{86}Rb^{+}$ influx and $^{86}Rb^{+}$ efflux were routinely monitored after either 5 or 10 min incubations. The addition of Oxo-M resulted in a stimulation of $^{86}Rb^{+}$ influx and $^{86}Rb^{+}$ efflux with EC_{50} values of 0.45 μM and 1.37 μM, respectively, and with Hill coefficients close to unity (Fig. 2.2). The inclusion of 10 μM atropine completely blocked Oxo-M-stimulation of both $^{86}Rb^{+}$ influx and $^{86}Rb^{+}$ efflux (data not shown).

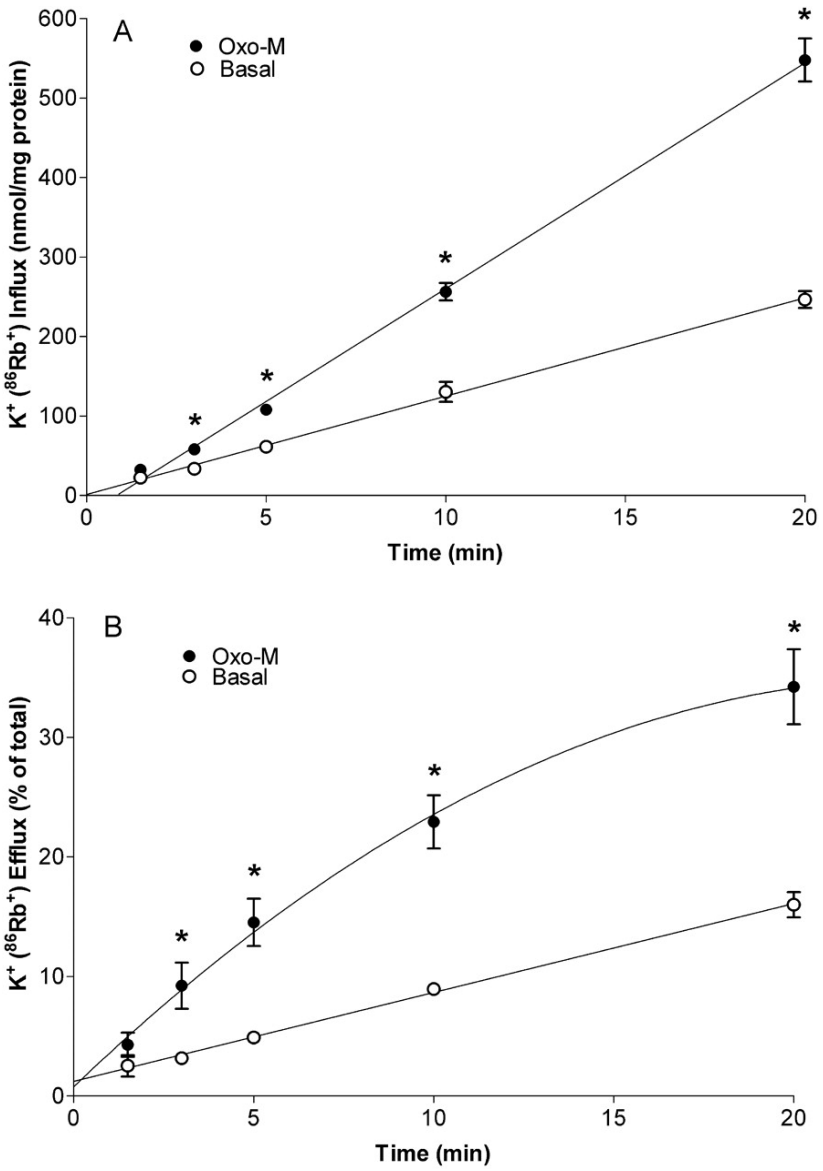

Figure 2.1 Kinetics of basal- and Oxo-M-stimulated $^{86}Rb^+$ influx and $^{86}Rb^+$ efflux. $^{86}Rb^+$ fluxes were monitored in SH-SY5Y cells incubated in hypotonic buffer A (230 mOsM) in the absence (○) or presence (●) of 100 µM Oxo-M. Results are expressed as either (A) K^+ ($^{86}Rb^+$) influx (nmol K^+ / mg protein; calculated assuming that $^{86}Rb^+$ uptake reflects that of K^+) or (B) K^+ ($^{86}Rb^+$) efflux (% of total radioactivity released). Values shown are the means ± S.E.M. for three independent experiments, each performed in triplicate. Where error bars are absent, the S.E.M. fell within the symbol. *, $p<0.05$, Different from basal (by unpaired Student's *t* test). Influx of $^{86}Rb^+$ at 340 mOsM was linear with time from 0-20 min, while efflux of $^{86}Rb^+$ was linear for at least 10 min when monitored at 340 mOsM.

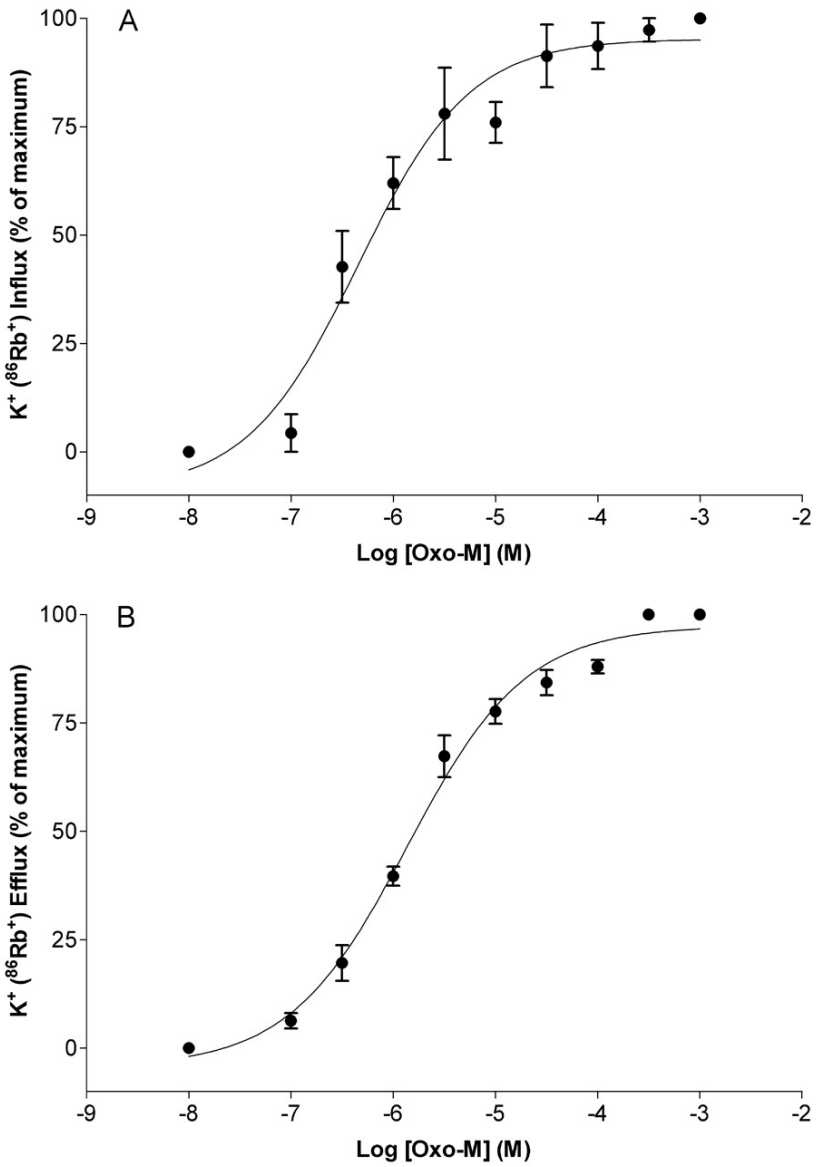

Figure 2.2 Dose-response relationships for Oxo-M-stimulated $^{86}Rb^+$ influx and $^{86}Rb^+$ efflux. $^{86}Rb^+$ influx (A) and $^{86}Rb^+$ efflux (B) were monitored in SH-SY5Y neuroblastoma cells incubated under hypotonic conditions (230 mOsM) in the presence or absence of Oxo-M at the concentrations indicated. Reactions were terminated after 10 min. Results are expressed as percentage of maximum agonist response (obtained at 1 mM Oxo-M) and are the means $\pm$ S.E.M. for three independent experiments, each performed in triplicate. Where error bars are absent, the S.E.M. fell within the symbol. The calculated EC_{50} value for $^{86}Rb^+$ influx was 0.45 µM with a Hill coefficient of 0.80. Addition of Oxo-M stimulated the efflux of $^{86}Rb^+$ with an EC_{50} of 1.37 µM and a Hill coefficient of 0.75.

Osmolarity dependence of basal- and Oxo-M-stimulated $^{86}Rb^{+}$ influx and $^{86}Rb^{+}$ efflux. Because the degree of facilitation of osmolyte release observed following mAChR activation has previously been demonstrated to be dependent on the extent of hypoosmotic stress (Loveday et al., 2003; Heacock et al., 2004), the ability of mAChR activation to regulate $^{86}Rb^{+}$ influx and $^{86}Rb^{+}$ efflux was monitored in SH-SY5Y cells under conditions of isotonicity (340 mOsM; defined by the osmolarity of the DMEM/fetal calf serum medium in which the cells were grown), mild-to-severe hypotonicity (310-200 mOsM), or mild hypertonicity (370 mOsM). Two experimental paradigms were employed to evaluate the dependence of $^{86}Rb^{+}$ fluxes on osmolarity. In the first, buffers were rendered either hypertonic or hypotonic by increases or decreases, respectively, in the NaCl concentration (since Na^{+} and Cl^{-} are the primary osmolytes found in plasma and reductions in plasma osmolarity observed under pathological conditions, such as hyponatremia, principally reflect changes in the concentrations of these ions). Under these conditions, the magnitude of basal $^{86}Rb^{+}$ influx was constant at all osmolarities tested. The addition of Oxo-M resulted in an increase in $^{86}Rb^{+}$ influx of ~ 75%, compared to basal, at all osmolarities (370-200 mOsM; Fig. 2.3A). In contrast, the basal efflux of $^{86}Rb^{+}$ was enhanced over that observed under isotonic conditions (340 mOsM) when osmolarity was reduced to 200 mOsM. Moreover, although the addition of Oxo-M resulted in a relatively small increase in $^{86}Rb^{+}$ efflux at both 340 and 370 mOsM, the extent of Oxo-M-stimulated $^{86}Rb^{+}$ efflux was significantly increased over isotonic at an osmolarity of 280 mOsM (a reduction in osmolarity of 18%) with a maximal enhancement observed at 230 mOsM (386% of basal; Fig. 2.3B). In the second

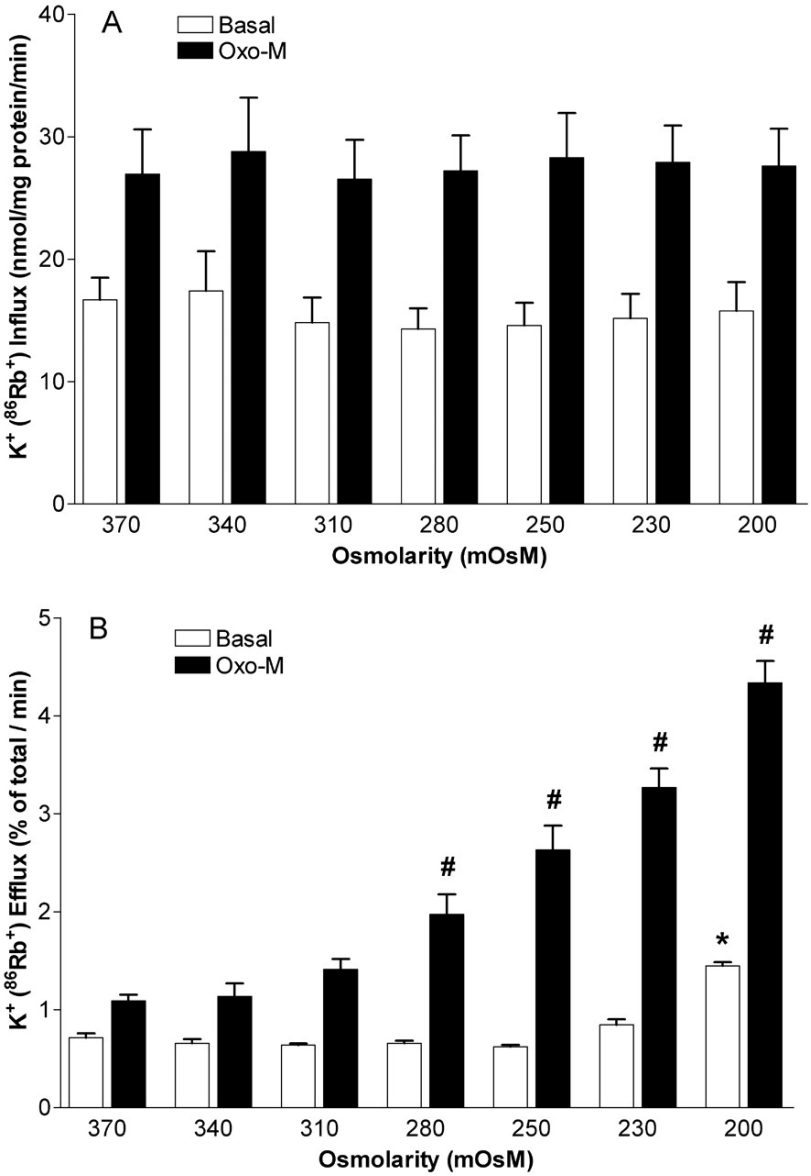

Figure 2.3 Basal- and Oxo-M-stimulated $^{86}Rb^{+}$ influx and $^{86}Rb^{+}$ efflux as a function of osmolarity. $^{86}Rb^{+}$ influx (A) and $^{86}Rb^{+}$ efflux (B) were monitored in SH-SY5Y neuroblastoma cells in buffers rendered hypertonic or hypotonic by an increase or decrease in NaCl concentration, respectively, in the absence (open bars) or presence (closed bars) of 100 μM Oxo-M. Reactions were terminated after 5 min and values shown are the means ± S.E.M. for three independent experiments, each performed in triplicate. *, $p<0.01$, Different from basal release monitored under isotonic (340 mOsM) conditions (by repeated measures ANOVA followed by Dunnett's multiple comparison test). #, $p<0.01$, Different from Oxo-M treatment under isotonic (340 mOsM) conditions (by repeated measures ANOVA followed by Dunnett's multiple comparison test). At all osmolarities, Oxo-M addition significantly increased both $^{86}Rb^{+}$ influx and $^{86}Rb^{+}$ efflux when compared to uptake or release monitored under basal conditions ($p<0.05$, by paired student's *t* test).

experimental paradigm, osmolarities of buffers were adjusted under conditions of a constant NaCl concentration (79 mM NaCl) by the addition of sucrose. Under these conditions, the basal influx of $^{86}Rb^{+}$ was significantly enhanced over that observed under isotonic conditions when the osmolarity was reduced to 230 or 200 mOsM (134% and 159% of that at 340 mOsM, respectively). The extent of Oxo-M–stimulated $^{86}Rb^{+}$ influx was also dependent upon osmolarity and, although an increased influx was monitored under isotonic conditions, significantly greater increases were observed at 230 and 200 mOsM than at 340 mOsM (Fig. 2.4A). The magnitudes of both basal- and Oxo-M-stimulated $^{86}Rb^{+}$ efflux were also found to be dependent upon the osmolarity of the buffer under conditions of a fixed concentration of NaCl and the values obtained for $^{86}Rb^{+}$ efflux were quantitatively similar for the two experimental paradigms (Fig. 2.4B).

$^{86}Rb^{+}$ influx is mediated primarily via Na^{+}/K^{+}-ATPase and the NKCC transporter under both basal and Oxo-M-stimulated conditions. K^{+} transport mechanisms, including Na^{+}/K^{+} ATPase and the Na^{+}-K^{+}-$2Cl^{-}$ (NKCC) and K^{+}-Cl^{-} (KCC) co-transporters, have previously been implicated in cell volume regulation. To determine the role, if any, played by these transporters in $^{86}Rb^{+}$ influx in SH-SY5Y neuroblastoma cells, both basal- and Oxo-M-stimulated $^{86}Rb^{+}$ influx were monitored in the absence or presence of pharmacological inhibitors at concentrations previously employed (Yabaluri and Medzihradsky, 1997; Ernest et al., 2005). Inclusion of 800 μM concentrations of either bumetanide or furosemide, inhibitors of the NKCC, attenuated both basal- and Oxo-M stimulated $^{86}Rb^{+}$ influx by ~50%.

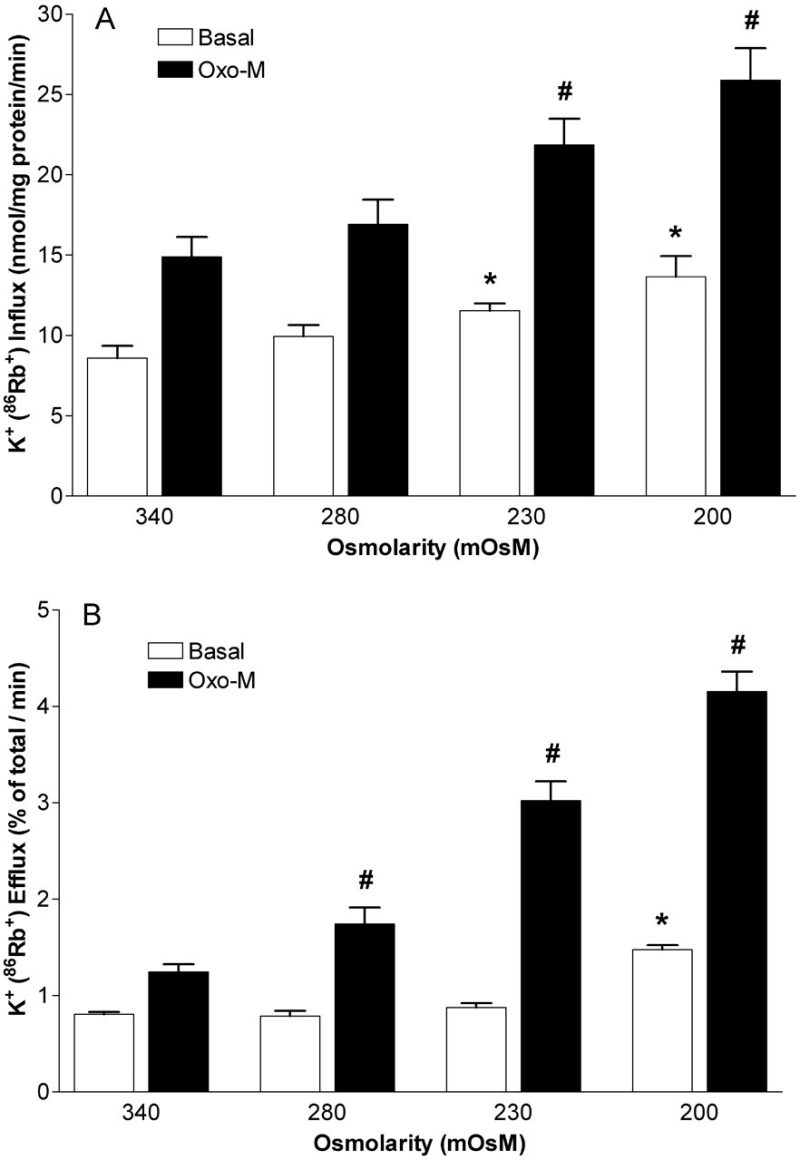

Figure 2.4 Basal- and Oxo-M-stimulated $^{86}Rb^+$ influx and $^{86}Rb^+$ efflux are volume-dependent. $^{86}Rb^+$ influx (A) and $^{86}Rb^+$ efflux (B) were monitored in SH-SY5Y neuroblastoma cells in buffers of the osmolarity indicated in the absence (open bars) or presence (closed bars) of 100 μM Oxo-M. Osmolarities of the buffers were adjusted under conditions of a constant NaCl concentration (79 mM NaCl) by the addition of sucrose. Reactions were terminated after 5 min of and results shown are the means ± S.E.M. for three or four independent experiments, each performed in triplicate. *, $p<0.05$, Different from basal influx or efflux monitored under isotonic (340 mOsM) conditions (by repeated measures ANOVA followed by Dunnett's multiple comparison test). #, $p<0.05$, Different from Oxo-M treatment under isotonic (340 mOsM) conditions (by repeated measures ANOVA followed by Dunnett's multiple comparison test). At all osmolarities, Oxo-M addition significantly increased both $^{86}Rb^+$ influx and $^{86}Rb^+$ efflux when compared to basal conditions ($p<0.05$, by paired student's *t* test).

Since furosemide inhibits both NKCC and KCC, we also evaluated the ability of DIOA, a KCC inhibitor, to attenuate $^{86}Rb^+$ influx. Inclusion of a 40 μM concentration of DIOA had no effect on basal $^{86}Rb^+$ influx but resulted in a 20% inhibition of the Oxo-M-mediated component. Inclusion of 30 μM ouabain, a selective inhibitor of the Na^+/K^+-ATPase, resulted in a significant inhibition (~40%) of both basal- and Oxo-M-stimulated $^{86}Rb^+$ influx. When both ouabain and furosemide were present, basal- and Oxo-M stimulated $^{86}Rb^+$ influx were essentially abolished (94% and 97% reductions, respectively; Fig. 2.5A). In contrast, neither the inclusion of bumetanide nor furosemide had any significant effect on basal $^{86}Rb^+$ efflux (Fig. 2.5B). Furthermore, the inclusion of the NKCC inhibitors resulted in either no effect (bumetanide) or a modest inhibition (19%, furosemide) when Oxo-M-stimulated $^{86}Rb^+$ efflux was monitored. Inclusion of DIOA also resulted in a small inhibition of Oxo-M-mediated $^{86}Rb^+$ efflux. Addition of ouabain had no effect on the magnitude of either basal- or Oxo-M-stimulated $^{86}Rb^+$ efflux, but when co-administered with furosemide, an inhibition of Oxo-M-stimulated $^{86}Rb^+$ efflux (~20%) was again observed (Fig. 2.5B). Taken collectively, these results suggest that $^{86}Rb^+$ influx and $^{86}Rb^+$ efflux are mediated by distinct mechanisms.

$^{86}Rb^+$ efflux is partially mediated by K^+ channels. Evidence that $^{86}Rb^+$ efflux is mediated, in part at least, by K^+ channels was obtained from experiments in which either the addition of TEA or Ba^{2+}, general inhibitors of K^+ channels, were found to attenuate both basal- and Oxo-M-stimulated efflux. Inclusion of TEA (10 mM) or Ba^{2+} (4 mM) significantly inhibited basal $^{86}Rb^+$ release by 37±7 and 38±6%,

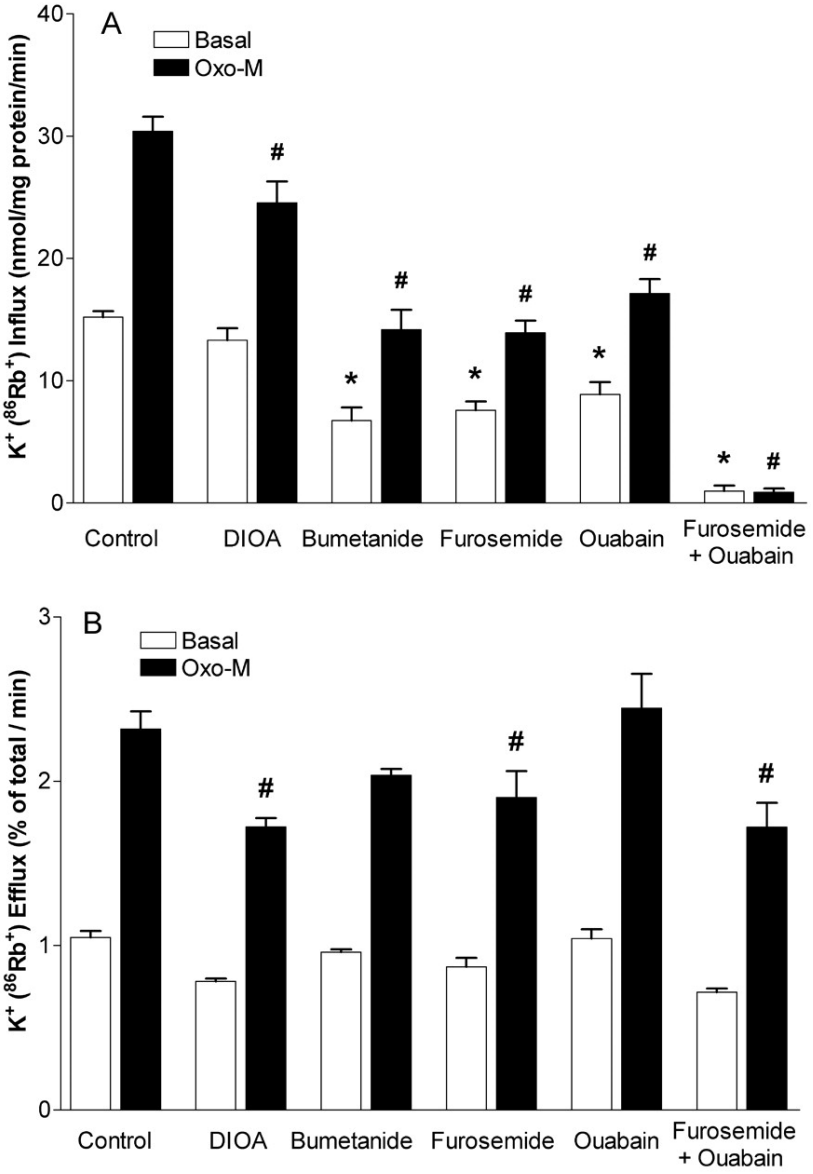

Figure 2.5 Effect of ouabain, DIOA, furosemide, or bumetanide on basal- and Oxo-M-stimulated $^{86}Rb^+$ influx and $^{86}Rb^+$ efflux. $^{86}Rb^+$ influx (A) and $^{86}Rb^+$ efflux (B) were monitored in SH-SY5Y neuroblastoma cells under hypotonic conditions (230 mOsM) in the absence (open bars) or presence (closed bars) of 100 μM Oxo-M. In some experiments, bumetanide (800 μM), furosemide (800 μM), DIOA (40 μM), and ouabain (30 μM) were also present. Reactions were terminated after 10 min and values shown are the means ± S.E.M. for three to nine independent experiments, each performed in triplicate. *, $p<0.05$, Different from control basal influx (by one-way ANOVA followed by Dunnett's multiple comparison test). #, $p<0.05$, Different from control plus Oxo-M (by one-way ANOVA followed by Dunnett's multiple comparison test).

respectively ($p<0.05$; n=6-9) and Oxo-M-stimulated $^{86}Rb^+$ efflux by 30±6 and 41±4% respectively ($p<0.05$; n=6-9). The addition of specific K^+ channel blockers such as apamin (1 μM) or iberiotoxin (100 nM), inhibitors of Ca^{2+}-activated channels, had little or no effect (<10%) on either basal or Oxo-M-stimulated $^{86}Rb^+$ efflux. Inclusion of glibenclamide (100 μM), an inhibitor of ATP-dependent K^+ channels, had no effect on basal $^{86}Rb^+$ release but did result in a small, yet significant reduction in Oxo-M-stimulated $^{86}Rb^+$ efflux (<15%).

Basal- and Oxo-M stimulated $^{86}Rb^+$ influx and $^{86}Rb^+$ efflux: dependence on Ca^{2+} availability and PKC activity. Previously we have demonstrated that the mAChR-stimulated osmosensitive release of the organic osmolyte, taurine, from SH-SY5Y cells is more dependent upon Ca^{2+} availability and PKC activity than that of the inorganic osmolyte, Cl^-, suggesting that the release of these osmolytes may be differentially regulated (Heacock et al., 2006a; Cheema et al., 2007). For this reason, in the present study, the roles played by Ca^{2+} availability and PKC activity in $^{86}Rb^+$ influx and $^{86}Rb^+$ efflux were evaluated. Although removal of extracellular Ca^{2+} had no effect on either basal- or Oxo-M-stimulated $^{86}Rb^+$ influx, the additional depletion of intracellular Ca^{2+} stores with 1 μM thapsigargin resulted in an increase in basal $^{86}Rb^+$ influx, whereas the ability of Oxo-M to enhance $^{86}Rb^+$ influx over the basal value was attenuated by approximately 35% under these conditions. Inclusion of 10 μM chelerythrine, a PKC inhibitor, had no effect on basal $^{86}Rb^+$ influx but significantly inhibited (50-60%) the Oxo-M-stimulated component, both in the presence or absence of Ca^{2+}/thapsigargin (Fig. 2.6A). Removal of extracellular Ca^{2+}

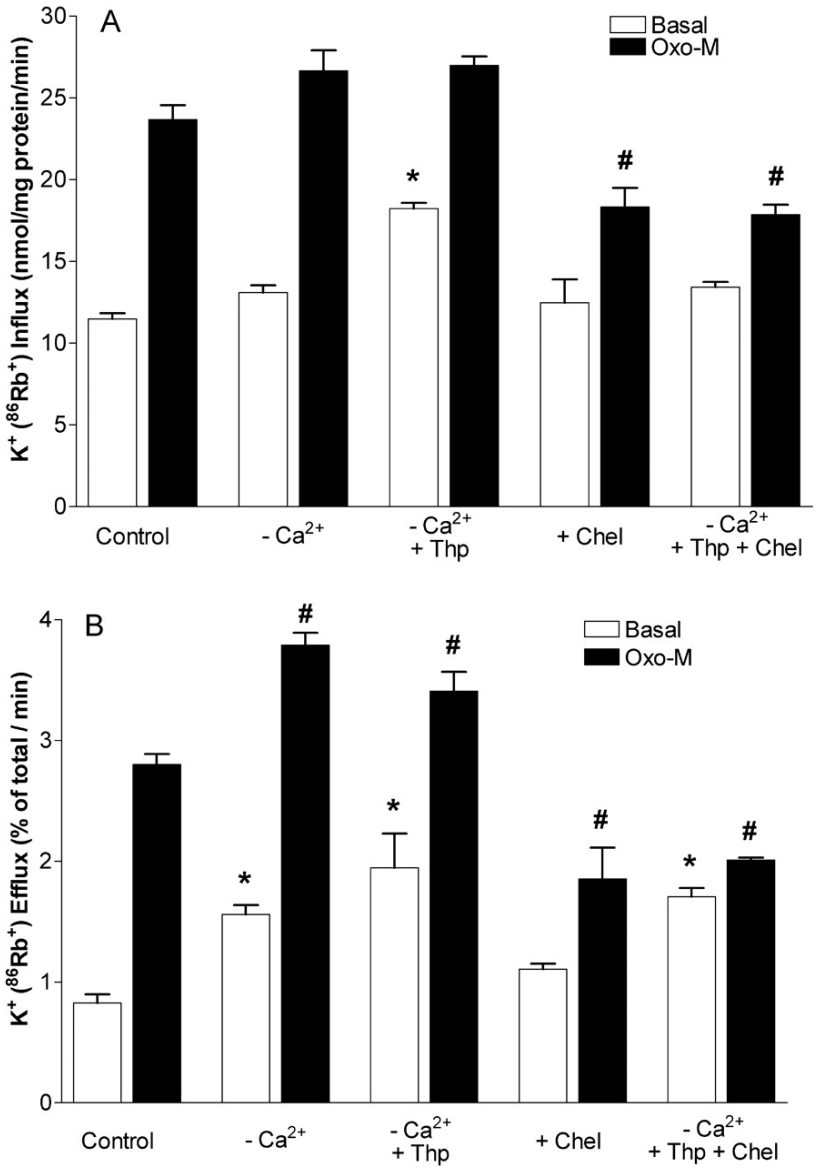

Figure 2.6 The role of extra- and intracellular Ca^{2+} and PKC in basal and Oxo-M-stimulated $^{86}Rb^+$ fluxes. $^{86}Rb^+$ influx (A) and efflux (B) were monitored in SH-SY5Y neuroblastoma cells under hypotonic conditions (230 mOsM). Cells were incubated in the absence (-Ca^{2+}: Ca^{2+} was omitted from buffer and 50 μM EGTA was added) or presence of extracellular Ca^{2+}. For some experiments, cells were pretreated in isotonic buffer for 15 min in the presence of 1 μM thapsigargin (Thp) to deplete intracellular pools of Ca^{2+}, and / or 10 μM of the PKC inhibitor chelerythrine. Reactions were allowed to proceed for 5 min in the absence (open bars) or presence (closed bars) of 100 μM Oxo-M. Results shown are the means ± S.E.M. for four to six independent experiments, each performed in triplicate. *, $p<0.05$, Different from control basal influx or efflux (by repeated measures ANOVA followed by Dunnett's multiple comparison test). #, $p<0.05$, Different from control plus Oxo-M (by repeated measures ANOVA followed by Dunnett's multiple comparison test).

resulted in an increase in basal efflux of $^{86}Rb^{+}$ whereas the ability of Oxo-M to increase efflux over the basal value was unchanged relative to control incubations. Depletion of intracellular Ca^{2+} stores with thapsigargin resulted in a further increase in the basal efflux of $^{86}Rb^{+}$ but significantly attenuated (~25%) the Oxo-M-mediated increase in $^{86}Rb^{+}$ efflux. As observed for $^{86}Rb^{+}$ influx, inclusion of chelerythrine had no effect on basal $^{86}Rb^{+}$ efflux but significantly inhibited (~60%) Oxo-M-mediated $^{86}Rb^{+}$ efflux. Under conditions in which intracellular stores of Ca^{2+} were depleted and PKC activity inhibited, the ability of Oxo-M to stimulate $^{86}Rb^{+}$ efflux was severely attenuated (86% inhibition, Fig. 2.6B).

Activation of multiple GPCRs can elicit both $^{86}Rb^{+}$ influx and $^{86}Rb^{+}$ efflux under hypotonic conditions. In addition to the mAChR, activation of several other GPCRs has been shown to increase the efflux of osmolytes from SH-SY5Y cells under hypotonic conditions (Heacock et al., 2006a; Cheema et al., 2007). These include the protease-activated receptor (PAR) which can be activated by thrombin and lysophospholipid receptors that can be selectively activated by either sphingosine-1-phosphate (S1P) or lysophosphatidic acid (LPA). To investigate whether activation of these receptors could also mediate changes in $^{86}Rb^{+}$ fluxes, thrombin (1.25 nM), S1P (5 μM) or LPA (10 μM) were added to SH-SY5Y cells under hypotonic conditions (230 mOsM) and $^{86}Rb^{+}$ influx and $^{86}Rb^{+}$ efflux monitored after a 10 min incubation. Addition of each of the three agonists resulted in a significant increase in both $^{86}Rb^{+}$ influx and $^{86}Rb^{+}$ efflux, with a rank order of efficacy for both fluxes being, thrombin = S1P > Oxo-M > LPA (Fig. 2.7). These observations can be

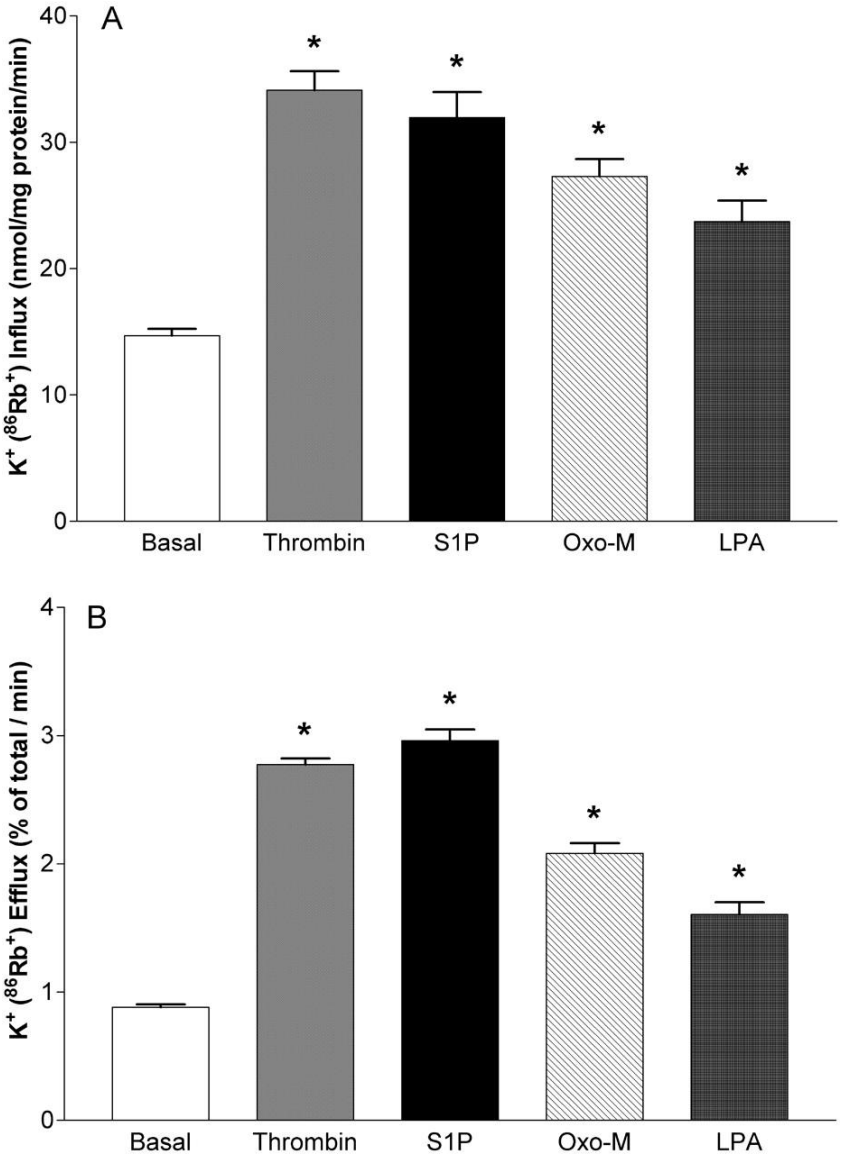

Figure 2.7 Activation of multiple GPCRs can induce both volume-dependent $^{86}Rb^{+}$ influx and $^{86}Rb^{+}$ efflux under hypotonic conditions. $^{86}Rb^{+}$ influx (A) and $^{86}Rb^{+}$ efflux (B) were monitored in SH-SY5Y neuroblastoma cells under hypotonic conditions (230 mOsM) in the presence of either 100 µM Oxo-M, 1.25 nM thrombin, 10 µM lysophosphatidic acid (LPA) or 5 µM sphingosine-1-phosphate (S1P). Reactions were terminated after 10 min and values shown are the means $\pm$ S.E.M. of four independent experiments, each performed in triplicate. *, $p<0.01$, Different from basal (by repeated measures ANOVA followed by Dunnett's multiple comparison test).

extended to glial cells since inclusion of either thrombin or S1P (but not Oxo-M) significantly enhanced both $^{86}Rb^+$ influx and $^{86}Rb^+$ efflux in primary cultures of rat astrocytes, when incubated under hypotonic conditions (Fig. 2.8).

Oxo-M-mediated regulation of intracellular K^+ concentration: roles of osmolarity and K^+ influx. The ability of Oxo-M to regulate the concentration of K^+ in SH-SY5Y cells under conditions of isotonicity (340 mOsM), mild hypotonicity (290 mOsM) or moderate hypotonicity (230 mOsM) was evaluated in the absence or presence of inhibition of K^+ influx following a 10 min incubation. Under isotonic conditions the intracellular K^+ concentration, as determined by means of a K^+-specific electrode, was 1.22 µmol K^+/mg protein, a value consistent with previous measurements of intracellular K^+ in the neuroblastoma 2A cell line (~1 µequiv. K+/mg protein: Kimelberg, 1974). Since the intracellular water space for SH-SY5Y cells was determined to be 8.5 µl/mg protein (n=3), the intracellular K^+ concentration of SH-SY5Y cells was calculated to be approximately 140 mM. Under either isotonic (340 mOsM) or mildly hypotonic (290 mOsM) conditions, the addition of Oxo-M had no significant effect on intracellular K^+ content. However, at both osmolarities, concurrent inhibition of K^+ influx by inclusion of furosemide (800 µM) and ouabain (30 µM) resulted in small, but significant reduction in K^+ mass under basal conditions (i.e. in the absence of the agonist). These reductions in K^+ concentration were accentuated by the presence of Oxo-M (Table 2.1). When cells were incubated under moderately hypotonic conditions (230 mOsM), the addition of Oxo- M resulted in a significant loss of K^+ from the cells, even when K^+ influx was operational.

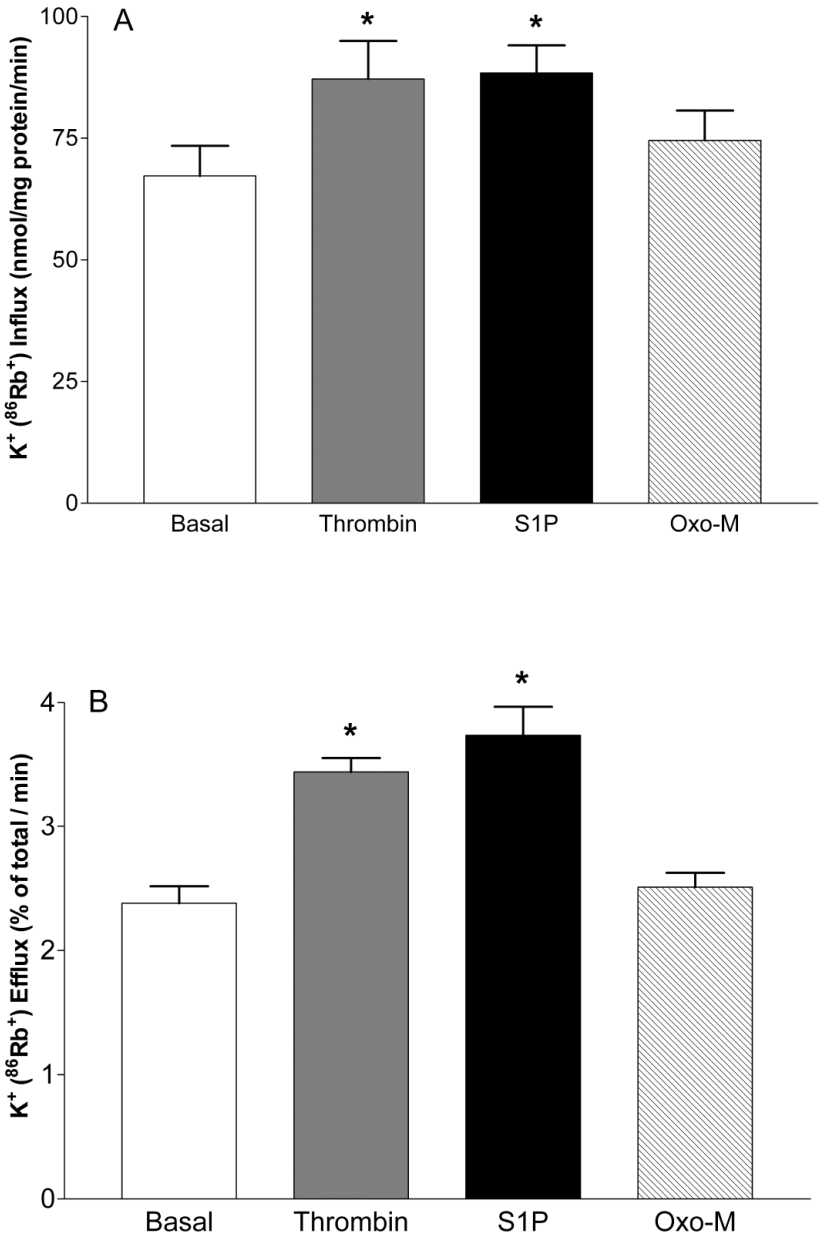

Figure 2.8 GPCR activation can induce both volume-dependent $^{86}Rb^{+}$ influx and $^{86}Rb^{+}$ efflux under hypotonic conditions in primary cultures of rat astrocytes. $^{86}Rb^{+}$ influx (A) and $^{86}Rb^{+}$ efflux (B) were monitored in primary cultures of rat astrocytes under hypotonic conditions (230 mOsM) in the presence or absence of 1.25 nM thrombin, 5 μM sphingosine-1-phosphate (S1P) or 100 μM Oxo-M. Reactions were terminated after 10 min and values shown are the means ± S.E.M. for three independent experiments, each performed in triplicate. *, $p<0.05$, Different from basal (by repeated measures ANOVA followed by Dunnett's multiple comparison test).

TABLE 2.1 Ability of Oxo-M, ouabain, and furosemide to regulate intracellular K^+ at different osmolarities. In three separate series of experiments, SH-SY5Y cells were exposed to either 340, 290 or 230 mOsM buffer A in the absence or presence of 100 μM Oxo-M, 30 μM ouabain and 800 μM furosemide. Reactions were terminated after 10 min and cells were washed with 2 ml of K^+ free isotonic buffer A. Cells were then lysed and intracellular K^+ content was measured using an ion specific electrode. Results are expressed as μmol K^+/mg protein. Results shown are the means ± S.E.M. for four independent experiments, each performed in duplicate. Percentage reductions from basal values are indicated in parentheses.

	Basal	+ Oxo-M	+ Ouabain + Furosemide	+ Oxo-M + Ouabain + Furosemide
340 mOsM	1.22 ± 0.02	1.21 ± 0.03	1.16 ± 0.02 (5%)*	1.12 ± 0.05 (8%)*#
290 mOsM	1.17 ± 0.03	1.16 ± 0.03	1.10 ± 0.04 (6%)*	1.03 ± 0.03 (12%)*#
230 mOsM	1.12 ± 0.08	1.01 ± 0.08 (10%)*	1.00 ± 0.08 (11%)*	0.86 ± 0.06 (23%)*#

*, $p<0.05$, Different from basal content (by repeated measures ANOVA followed by Dunnett's multiple comparison test).
#, $p<0.05$, different from both Oxo-M treatment and ouabain / furosemide treatment (by repeated measures ANOVA followed by Dunnett's multiple comparison test).

However, when K^+ influx was prevented by inclusion of furosemide and ouabain, at this osmolarity the addition of Oxo-M resulted in a further loss of cell K^+ (23%) during a 10 min incubation period (Table 2.1). This value for loss of K^+ agrees closely with measurement of $^{86}Rb^+$ efflux under Oxo-M-stimulated conditions (rate = 2.61 % per min: Fig. 2.1B).

Discussion

Previous studies of osmolyte loss from neural cells following their exposure to a hypoosmotic medium have focused almost exclusively on measurement of efflux. However, the net loss of an osmolyte from a cell reflects both its release and uptake. The principal finding to emanate from the present study is that, under hypoosmotic conditions, the activation of mAChRs in SH-SY5Y neuroblastoma cells can enhance the osmosensitive efflux **and** influx of $^{86}Rb^{+}$. Furthermore, these two fluxes exhibit similar characteristics in terms of kinetics, agonist concentration-dependence and requirements for Ca^{2+} availability and PKC activity. Because SH-SY5Y cells exhibit a relatively homogeneous population of M3 mAChRs (>80% of total: Wall et al., 1991; Slowiejko et al., 1994), it is likely that this mAChR subtype mediates the increase in $^{86}Rb^{+}$ fluxes, as has been previously demonstrated for the osmosensitive release of taurine (Heacock et al., 2004). In addition to the mAChR, activation of several other GPCRs that have previously been demonstrated to regulate the osmosensitive release of taurine from SH-SY5Y cells, i.e. PAR-1, S1P and LPA receptors (Heacock et al., 2006a; Cheema et al., 2007), also promoted both the efflux and influx of $^{86}Rb^{+}$ under hypoosmotic conditions (Fig. 2.7). Furthermore, activation of PAR-1 and S1P receptors also facilitated both the influx and efflux of $^{86}Rb^{+}$ in primary cultures of rat astrocytes (Fig. 2.8). Taken collectively, these results suggest that the ability of GPCRs to regulate both the influx and efflux of $^{86}Rb^{+}$ under hypoosmotic conditions may be a general property of neural cells. Although Oxo-M-stimulated efflux and influx of $^{86}Rb^{+}$ share similar characteristics, the two fluxes

are mediated via distinct mechanisms. Under hypoosmotic conditions, both basal- and Oxo-M-mediated $^{86}Rb^{+}$ influx are abolished following administration of ouabain and furosemide, a result that indicates that $^{86}Rb^{+}$ influx is mediated principally through the Na^{+}-K^{+}-$2Cl^{-}$ cotransporter (NKCC) and Na^{+}/K^{+}-ATPase (Fig. 2.5A). These findings are consistent with previous studies in which the basal influx of $^{86}Rb^{+}$ in astrocytes and C6 glioma cells, monitored under isoosmotic or hypoosmotic conditions, was also inhibited by ouabain and furosemide (Kimelberg and Frangakis, 1985; Mongin et al., 1994; Mongin et al., 1996). KCC may also play a minor role in $^{86}Rb^{+}$ influx in SH-SY5Y cells, as previously suggested for C6 glioma (Gagnon et al., 2007). In contrast to the results obtained for $^{86}Rb^{+}$ influx, none of the agents tested had any effect on basal $^{86}Rb^{+}$ efflux and only the addition of either furosemide or DIOA resulted in a small inhibition (<20%) of the Oxo-M-mediated component, a result consistent with a limited involvement of KCC in $^{86}Rb^{+}$ efflux (Fig. 2.5B). However, the majority of $^{86}Rb^{+}$ efflux appears to be mediated by K^{+} channels since inclusion of either TEA or Ba^{2+}, general inhibitors of K^{+} channels, attenuated both basal- and Oxo-M-stimulated efflux. However, since charybdotoxin (100 nM), apamin (1 μM), iberiotoxin (100 nM) and glybenclamide (100 nM) (inhibitors of Ca^{2+}-activated and ATP-dependent channels) had no significant inhibitory effect on $^{86}Rb^{+}$ efflux, the identity of the specific K^{+} channel(s) involved remains to be determined (data not shown).

The magnitude of basal- and Oxo-M-stimulated efflux of taurine and $^{125}I^{-}$ (used as a tracer for Cl^{-}) from SH-SY5Y cells is dependent upon the degree of osmotic stress, when monitored under conditions in which the buffers are rendered

increasingly hypotonic by a reduction in NaCl concentration (Heacock et al., 2004; Cheema et al., 2007). However, under the same conditions, basal- and Oxo-M-stimulated $^{86}Rb^{+}$ influx appeared to be independent of osmolarity (Fig. 2.3A). Although this experimental paradigm mimics the changes encountered under physiological conditions, it also involves alterations in three experimental variables, i.e. osmolarity and the concentrations of Na^{+} and Cl^{-} ions. When monitored under conditions in which NaCl concentration was held constant and osmolarity varied by means of the addition of sucrose, it was evident that the magnitude of both basal- and Oxo-M-stimulated $^{86}Rb^{+}$ influx was dependent upon osmolarity (Fig. 2.4A). Both basal- and Oxo-M-stimulated efflux of $^{86}Rb^{+}$ were found to be dependent upon the degree of osmolarity, regardless of which experimental paradigm was employed (Fig. 2.3). Thus, we conclude that whereas $^{86}Rb^{+}$ efflux occurs via an osmolarity-sensitive, but NaCl-independent mechanism, $^{86}Rb^{+}$ influx is mediated by a mechanism that is dependent on both osmolarity and NaCl, consistent with the involvement of NKCC and Na^{+}/K^{+}-ATPase. Although both the influx and efflux of $^{86}Rb^{+}$ in SH-SY5Y cells are osmosensitive, the efflux component is more dependent on changes in osmolarity, as is evident from the observation that whereas the Oxo-M-mediated component of $^{86}Rb^{+}$ influx doubles when osmolarity is reduced from 340 to 200 mOsM, the corresponding increase for $^{86}Rb^{+}$ efflux is 6-7-fold (Fig. 2.4).

The osmosensitive efflux of taurine and $^{125}I^{-}$ from SH-SY5Y cells following activation of mAChRs (but not that monitored under basal conditions) is differentially regulated, with the efflux of $^{125}I^{-}$ exhibiting less dependence on Ca^{2+} availability and PKC activity than that observed for taurine (Cheema et al., 2007). Thus, whereas

removal of extracellular Ca^{2+} attenuates mAChR-stimulated taurine efflux by >60%, and depletion of intracellular Ca^{2+} abolishes the response, $^{125}I^-$ efflux is unaffected by removal of extracellular Ca^{2+} and only minimally reduced by depletion of intracellular Ca^{2+} (~30%). Similarly, mAChR-stimulated taurine efflux is more susceptible to inhibition of PKC than is that of $^{125}I^-$ release (Heacock et al., 2006a; Cheema et al., 2007). In the current study, the Ca^{2+} requirements observed for $^{86}Rb^+$ influx and efflux resembled more closely those previously obtained for $^{125}I^-$ release than for taurine efflux. Thus removal of extracellular Ca^{2+} had no effect on the magnitude of either mAChR-stimulated $^{86}Rb^+$ influx or efflux and only under conditions in which the intracellular pool of Ca^{2+} was depleted was the Oxo-M-mediated component reduced by 25-35% (Fig. 2.6). In contrast, both the basal influx and efflux of $^{86}Rb^+$ were increased by removal of Ca^{2+}. An increase in $^{86}Rb^+$ efflux under Ca^{2+}-depleted conditions has also been observed for astrocytes, although the mechanism remains unclear (Quesada et al., 1999). Oxo-M-stimulated $^{86}Rb^+$ influx and efflux were also dependent on PKC activity and could be attenuated by ~50% following pre-incubation of the cells with chelerythrine. From this series of experiments, two conclusions can be drawn. First, the differential Ca^{2+} requirements observed for basal- and mAChR-stimulated $^{86}Rb^+$ release provide additional support for the proposal that distinct mechanisms underlie the swelling-activated and receptor-mediated components of osmolyte release (Mongin and Kimelberg, 2005; Heacock et al., 2006a). Second, since both Oxo-M-mediated efflux of $^{86}Rb^+$ and $^{125}I^-$ exhibit requirements for Ca^{2+} and PKC activity that are distinct from those necessary for taurine release, these results indicate that the receptor-mediated release of

inorganic ($^{86}Rb^+$ and $^{125}I^-$) and organic osmolytes (taurine) occurs via distinct mechanisms.

Although the use of the radiotracer, $^{86}Rb^+$, provides a convenient means whereby the characteristics of K^+ influx and efflux pathways are readily evaluated, this approach does not permit a quantitative assessment of the relative contributions made by each pathway to K^+ content of cells. To address this issue, we monitored changes in the concentration of K^+ in SH-SY5Y cells under hypoosmotic conditions using a K^+-specific electrode. The results indicated that the addition of Oxo-M had no effect on the K^+ content of SH-SY5Y cells when exposed to either isoosmolarity or a mild reduction in osmolarity (290 mOsM) unless K^+ influx was concurrently prevented by inclusion of ouabain and furosemide (Table 2.1). This result suggests that under normal conditions, the agonist stimulation of K^+ efflux is countered by an equivalent stimulation of K^+ influx. When monitored under more hypoosmotic conditions (230 mOsM), Oxo-M addition results in a 10% reduction of K^+ content and this loss is further accentuated when K^+ influx is prevented (23% reduction). The results obtained from measurement of K^+ content are consistent with those derived from radiolabeling studies which indicate that although both K^+ influx and efflux are osmosensitive, it is the efflux pathway that is most strongly regulated by a reduction in osmolarity (Fig. 2.4). Thus, although an increase in K^+ efflux is offset by a comparable increase in K^+ influx under conditions of limited reductions in osmolarity, when cells are incubated under more hypoosmotic conditions, the efflux of K^+ predominates and a net loss of K^+ occurs.

Previous studies have indicated that, under conditions of chronic hyponatremia, the brain selectively retains its inorganic osmolytes (Melton et al., 1987; Pasantes-Morales et al., 2002b; Massieu et al., 2004). In the present study, we have demonstrated that activation of GPCRs not only enhances the efflux of K^+, but also its influx. Under conditions of limited reductions in osmolarity, such as those most likely to occur under pathological conditions, the basal uptake of K^+, which is mediated via the NKCC and Na^+/K^+-ATPase, is markedly facilitated by receptor activation and this permits K^+ to be more effectively retained by the cells. These results raise the possibility that, under hyponatremic conditions, tonic receptor activity in the CNS may serve not only to enhance the efflux of osmolytes, but also to maintain relatively high intracellular concentrations of K^+.

Chapter 3

Muscarinic Receptor Regulation of Osmosensitive Taurine Transport in Human SH-SY5Y Neuroblastoma Cells

Summary

The ability of G-protein-coupled receptors to regulate osmosensitive uptake of the organic osmolyte, taurine, into human SH-SY5Y neuroblastoma cells has been examined. When monitored under isotonic conditions and in the presence of physiologically relevant taurine concentrations (1-100 μM), taurine influx was mediated exclusively by a Na^+-dependent, high-affinity (K_m=2.5 μM) saturable transport mechanism (V_{max}=0.087 nmol/mg protein/min). Reductions in osmolarity of >20% (attained under conditions of a constant NaCl concentration) resulted in an inhibition of taurine influx (>30%) that could be attributed to a reduction in V_{max}, whereas the K_m for uptake remained unchanged. Inclusion of the muscarinic cholinergic agonist, oxotremorine-M (Oxo-M), also resulted in an attenuation of taurine influx (EC_{50}~0.7 μM). Although Oxo-M-mediated inhibition of taurine uptake could be observed under isotonic conditions (~25-30%), the magnitude of inhibition was significantly enhanced by hypotonicity (~55-60%), a result that also reflected a

reduction in the V_{max}, but not the K_m, for taurine transport. Oxo-M-mediated inhibition of taurine uptake was dependent upon the availability of extracellular Ca^{2+} but was independent of protein kinase C activity. In addition to Oxo-M, inclusion of either thrombin or sphingosine 1-phosphate also attenuated volume-dependent taurine uptake. The ability of Oxo-M to inhibit the influx of taurine was attenuated by 4-[(2-butyl-6,7-dichloro-2-cyclopentyl-2,3-dihydro-1-oxo-1*H*-inden-5-yl)oxy]butanoic acid (DCPIB), an inhibitor of the volume-sensitive organic osmolyte and anion channel. DCPIB also prevented receptor-mediated changes in the efflux and influx of K^+ under hypoosmotic conditions. The results suggest that muscarinic receptor activation can regulate both the volume-dependent efflux and uptake of taurine and that these events may be functionally coupled.

Introduction

When exposed to hypoosmotic stress, cells initially swell in proportion to the reduction in osmolarity and then normalize their volume via a process known as regulatory volume decrease. The latter involves the release of osmolytes, both inorganic (K^+, Cl^-) and organic (amino acids, polyols and methylamines) and the exit of obligated water (McManus et al., 1995). Although inorganic osmolytes constitute the quantitatively major fraction of the osmolyte pool (60-65%; Pasantes-Morales et al., 2002b), large changes in ion concentration can adversely impact cell excitability. Thus the pool of 'non-perturbing' organic osmolytes plays a pivotal role in countering changes in osmolarity without compromising cell function. One of the most quantitatively important organic osmolytes in the CNS is the sulfur amino acid, taurine (Huxtable, 1992; Miller et al., 2000; Lambert, 2004). In response to hyposmolarity, taurine is primarily released from neural cells via a volume-sensitive organic osmolyte and anion channel (VSOAC), although the involvement of additional channels cannot be discounted (Shennan, 2008). VSOAC, which has been extensively characterized both electrophysiologically and pharmacologically, is selectively permeable to anions (primarily Cl^-) and organic osmolytes. However, despite intensive efforts, the molecular identity of VSOAC remains unknown (Okada, 2006).

The most common cause of hypoosmotic swelling is a condition known as hyponatremia, which is defined as a reduction in serum Na^+ concentration below a normal value of 145 to 136 mEq/L . In severe cases of hyponatremia, serum Na^+

concentrations of < 110 mEq/L have been reported, which represent a reduction in plasma osmolarity of ~25% (Haussinger et al., 1994). Hyponatremia is the most commonly encountered electrolyte disorder in clinical practice and is conservatively estimated to occur in 2.5-5.0% of hospitalized patients, with the young and elderly disproportionately affected (Bhardwaj, 2006; Lien and Shapiro, 2007). The majority of symptoms associated with hyponatremia are neurological in origin and these include nausea, headache, coma and respiratory arrest. Reductions in the concentrations of brain organic osmolytes during hyponatremia can be quite dramatic. Thus, when animals are rendered chronically hyponatremic, substantial losses of the major organic osmolytes (taurine, glutamate and myo-inositol) from the CNS are observed with taurine being the most severely impacted (70-90% reductions; Lien et al., 1991; Pasantes-Morales et al., 2002b; Massieu et al., 2004).

The volume-dependent efflux of taurine from a variety of neural preparations in response to hyposmolarity has been extensively documented. In addition, the magnitude of osmosensitive taurine release may be further enhanced following the activation of specific GPCRs (for review, see Fisher et al., 2008). However, it is less clear whether the uptake of taurine, which, under physiologically relevant concentrations, is mediated primarily via a Na^+-dependent, high-affinity transport system (TauT), is also subject to such regulation. For example, whereas high affinity taurine transport is reported to be unaffected by a reduction in osmolarity in either astrocytes or cerebellar granule cells (Sanchez-Olea et al., 1991; Schousboe et al., 1991), a recent study indicated that taurine uptake into rat hippocampal neurons was significantly attenuated in response to a reduction in osmolarity (Olson

and Martinho, 2006). A volume-dependent regulation of osmolyte uptake could potentially have a significant impact on osmolyte homeostasis since the net loss of an osmolyte from a hypoosmotically stressed cell would reflect not only its efflux, but also its re-uptake. In this context, we recently observed that under hypoosmotic conditions, the activation of specific GPCRs on human SH-SY5Y neuroblastoma cells resulted not only in an increase in the efflux of K^+ (monitored as $^{86}Rb^+$), but also in its influx (Foster et al., 2008). These opposing effects serve to minimize the loss of K^+ from hypoosmotically-stressed cells. In the present study, we have examined the possibility that taurine uptake into SH-SY5Y cells may also be subject to regulation by osmolarity and receptor activation. The results indicate that, in contrast to the situation pertaining to K^+ influx, hyposmolarity *attenuates* the high affinity uptake of taurine into these cells and that receptor activation elicits a further inhibition of transport. Inhibition of taurine uptake could be largely prevented when osmosensitive taurine efflux was blocked by inclusion of DCPIB, an inhibitor of VSOAC. Thus under conditions of hypotonicity, activation of GPCRs on SH-SY5Y cells elicits both a facilitation of taurine efflux and an attenuation of taurine uptake, regulatory events which act in concert to enhance the loss of the osmolyte from cells.

Materials and Methods

Materials: [1,2-^{3}H]Taurine (1.15 TBq/mmol) and myo-[2-^{3}H]inositol 2.4 TBq/mmol) was obtained from GE Healthcare (Chalfont St. Giles, UK). Rubidium chloride ($^{86}Rb^{+}$-labeled: 37 GBq/g) was obtained from PerkinElmer Life and Analytical Sciences (Waltham, MA). Oxotremorine-M, sphingosine 1-phosphate, thrombin, PMA, β-alanine, hypotaurine and atropine were purchased from Sigma-Aldrich (St. Louis, MO). Guanidinethyl sulfonate was purchased from Toronto Chemicals (Toronto, ON). DCPIB was obtained from Tocris Bioscience, Inc. (Ellisville, MO). Thapsigargin, Ro-31-8220, Gö 6983 and bisindolylmaleimide 1 (BIM) were obtained from Calbiochem (San Diego, CA). Dulbecco's modified Eagle medium (DMEM) and 50x penicillin/streptomycin were obtained from Invitrogen (Carlsbad, CA). Fetal calf serum was obtained from Lonza Walkersville, Inc. (Walkersville, MD). Tissue culture supplies were obtained from Corning Inc. (Corning, NY), Starstedt (Newton, NC) and BD BioSciences (San Jose, CA). Universol was obtained from MP Biomedicals (Solon, OH).

Cell Culture: Human SH-SY5Y neuroblastoma cells (passages 70-89) were grown in tissue culture flasks (75 cm^2/250mL) in 20 mL of DMEM supplemented with 10% (v/v) of fetal calf serum with 1% penicillin/streptomycin. The osmolarity of the medium was 330-340 mOsM. Cells were grown at 37^{o}C in a humidified atmosphere containing 10% CO_2. The medium was aspirated and cells detached from the flask with a trypsin-versene mixture (Cambrex Bio Science, Walkersville, MD). Cells were

then resuspended in DMEM/10% fetal calf serum with penicillin/streptomycin and subcultured into 35-mm, six-well culture plates at a density of 250-300,000 cells/well for 4 to 5 days. Cells that had reached 70-90% confluence with a protein content of ~0.25 mg protein per well were routinely used.

Measurement of taurine influx: SH-SY5Y neuroblastoma cells were washed twice with 2 mL of isotonic buffer (142mM NaCl, 5.6 mM KCl, 2.2 mM $CaCl_2$, 3.6 mM $NaHCO_3$, 1 mM $MgCl_2$, 30 mM HEPES, pH 7.4, and 1 mg/mL D-glucose, ~340 mOsM). Unless specified otherwise, cells (~0.25 mg protein) were then incubated for 10 min at 37^{o}C in buffer (380-230 mOsM) that contained 5 μM [^{3}H]taurine (0.1-0.2 μCi/mL) in the presence or absence of agonist. A potential complication of the measurement of both basal- and Oxo-M-mediated taurine uptake under hypoosmotic conditions is that a concurrent release of endogenous taurine from the cells might lower the specific activity of [^{3}H]taurine. To minimize the contribution of taurine released from cells during measurements of taurine uptake, a 5 mL assay volume (25 nmol of added taurine) was routinely employed. From measurement of taurine contents in SH-SY5Y cells (see Results) and assuming a 20-30% release of taurine during a 10 min incubation under receptor-stimulated conditions (Heacock et al., 2004), it can be calculated that the maximal contribution from release of endogenous taurine in each well is ~3-4 nmol (a potential 11-14% reduction in specific activity). However this value is likely to be an over-estimate since it assumes that taurine is released instantaneously from cells, whereas receptor-mediated efflux of taurine has been demonstrated to occur in a time-dependent manner during the 10 min

incubation (Heacock et al., 2004). Two additional series of experiments were conducted to further assess the possibility that the release of endogenous taurine might complicate interpretation of the results. In the first, basal- and Oxo-M-mediated changes in taurine uptake were monitored in the presence of an increased assay volume (10 mL), conditions under which a contribution from endogenous taurine would be reduced. However, the rates of taurine uptake under both basal and agonist-stimulated conditions were indistinguishable when monitored in 5 or 10 mL volumes and the addition of Oxo-M resulted in 52 ± 2 and 49 ± 2 % inhibition of uptake, respectively ($p<0.01$ vs basal, $n=4$). In a second series of experiments, we used to advantage the observations that (i) mAChR-mediated inhibition of taurine influx does not readily desensitize (see Fig. 3.1A) and (ii) the mAChR-sensitive pool of taurine is readily releasable. Thus cells were first preincubated for 5 min at 37^{o}C in the presence of 100 μM Oxo-M in hypoosmotic buffer (230 mOsM), conditions under which ~75% of the mAChR-sensitive pool of taurine is released (Heacock et al., 2006a). Following the preincubation period, the medium was aspirated and cells washed with 2 mL of hypotonic buffer to remove residual taurine. Cells were then incubated for an additional 20 min in hypotonic buffer at 37^{o}C in the presence or absence of Oxo-M and taurine uptake monitored following the addition of [^{3}H]taurine. Under these conditions, the addition of Oxo-M also resulted in a marked inhibition of taurine uptake (43 ± 4 %, $p<0.01$ vs basal, $n=5$). In cells prelabeled to equilibrium with [^{3}H]taurine, the rate of [^{3}H]taurine efflux monitored during the 5 min preincubation period under Oxo-M-stimulated conditions exceeded that observed during the subsequent 20 min incubation period by ~10-fold (3.93 ± 0.30 vs. 0.37 ±

0.007 percent of initial radioactivity released/min, respectively, n=4, $p<0.01$). Both series of experiments indicate that the release of endogenous taurine does not have a significant impact on the measurement of rates of [^{3}H]taurine uptake.

Throughout the present study a 5 mL assay volume was routinely employed except when taurine concentrations of <5 μM were utilized (as for the substrate-dependence studies shown in Fig. 3.4). In those experiments, the assay volume was increased to 10 mL to reduce the possibility of a significant dilution of the specific activity when the lower concentrations of taurine were employed. To terminate the reactions involving measurement of taurine uptake, the extracellular medium was aspirated, cells rapidly washed with 2 mL of isotonic buffer A and lysed with 2 mL of 0.1 M NaOH. Aliquots of lysate (1 mL) were removed and radioactivity determined after the addition of 6.5 mL of Universol scintillation fluid. In all measurements, radioactivity associated with the cells at the zero time point was subtracted from the observed values. Except when indicated otherwise, the osmolarity of buffers was routinely adjusted under conditions of a constant NaCl concentration (95 mM NaCl) by the addition of sucrose. In some experiments, buffers were rendered either hypertonic or hypotonic by an increase or decrease in NaCl concentration, respectively. Osmolarities of buffers were monitored by means of an Osmette Precision osmometer (PS Precision Systems, Sudbury, MA). Protein contents of cell lysates were determined using a bicinchoninic acid protein assay reagent kit (Thermo Scientific; Rockford, IL). From the measurement of [^{3}H]taurine uptake, rates of taurine influx were calculated as nmol/mg protein/min. Throughout

the study, “basal” uptake of taurine is defined as that which occurs at a specified osmolarity in the absence of an agonist.

Measurement of taurine efflux: Taurine efflux from SH-SY5Y cells was monitored essentially as previously described (Heacock et al., 2004) . In brief, cells were prelabeled to isotopic equilibrium with 0.25 μCi/ mL of [^{3}H]taurine at 37^{o}C for 24 h. After prelabeling, the cells were washed twice with 2 mL of isotonic buffer A (~340 mOsM) and then incubated at 37^{o}C in 2 mL of buffer A (370-230 mOsM) in the absence or presence of agonist. The osmolarity of buffer A was adjusted under conditions of a constant NaCl concentration (95 mM NaCl) by the addition of sucrose. After 10 min of incubation, aliquots (1 mL) of the extracellular medium were removed and radioactivity determined after the addition of 6.5 mL of Universol scintillation fluid. The reactions were terminated by rapid aspiration of the remaining medium, and cells were lysed by the addition of 2 mL of 6% trichloroacetic acid. Taurine efflux was calculated as a fractional release, i.e., the radioactivity released into the extracellular medium as a percentage of the total radioactivity present initially in the cells. The latter was calculated as the sum of radioactivity recovered in the extracellular buffer and that remaining in the lysate at the end of the assay. ‘Basal’ efflux of taurine is defined as that which occurs at a specified osmolarity in the absence of an agonist.

Determination of taurine mass: Taurine concentrations were determined following *o*-phthaldialdehyde (OPA) derivatization, essentially as previously described

(Canevari et al., 1992; Alvarez et al., 2006). SH-SY5Y cells were washed with 2 x 2 mL of isotonic buffer A and then lysed in 2 mL of ice cold 6% TCA. Cell extracts were neutralized (pH 7-8) by addition of K_2CO_3 and diluted 1:1 with sodium borate buffer (0.2 M, pH 9.6). A 50 µl aliquot of each sample was removed and derivatized with 25 µl of OPA solution (15 mM OPA, 30 mM 2-mercaptoethanol, and 10% methanol in 0.2 M sodium borate buffer, pH 9.6) in an autosampler (Agilent 1100 series) for 1 min at 10^{o}C. A 10 µl aliquot of the derivatized sample was then injected into the HPLC column (ZORBAX Eclipse XDB-C18 (5 µm) analytical column 4.6 x 150 mm) and eluted at a flow rate of 1 ml/min with buffers A (80% 0.1 M sodium acetate and 20% methanol, pH 4.75) and B (20% 0.1 M sodium acetate and 80% methanol, pH 4.75). The following increasing gradient of buffer B was used for elution: 0-10 min, 30 to 60%; 10-15 min, 60 to 100%; 15-20 min, 100%; 20-22 min, 100 to 30%; 22-30 min, 30%, with a corresponding decrease in the percentage of buffer A. The detector was set at 340 nm excitation and 450 nm emission wavelengths, taurine concentrations were determined using calibration coefficients obtained with standard taurine solutions. Protein contents of cell lysates were determined using a bicinchoninic acid protein assay reagent kit (Thermo Scientific; Rockford, IL) and values for intracellular taurine content in SH-SY5Y cells were reported as nmol / mg protein. To determine the taurine content in the cell culture medium, DMEM supplemented with 10% fetal calf serum was mixed 1:1 with 10% TCA and the resulting protein precipitate was removed by centrifugation. The supernatant was neutralized to pH 7-8 by addition of K_2CO_3 and diluted 1:4 with 0.2

M sodium borate buffer (pH 9.6) prior to OPA-derivatization and subsequent HPLC analysis as outline above.

Data Analysis: All experiments shown were performed in duplicate or triplicate and repeated at least three times. Values quoted are given as means ± SEM for the number (n) of independent experiments indicated. A two-tailed Student's t test (paired or unpaired) was used to evaluate differences between two experimental groups (level of significance, $p < 0.05$). Ordinary or repeated measures analysis of variance (ANOVA) followed by Dunnett's multiple comparisons test was used for statistical significance of differences between multiple groups. EC_{50} values were obtained using Prism 5.0 (GraphPad Software Inc., San Diego, CA). Kinetic analysis of taurine uptake data was performed as previously reported (Schousboe et al., 1976; Olson and Martinho, 2006). Data were fit to the sum of carrier-mediated transport and diffusional components using a non-linear least squares approximation according to the following equation (Equation 1):

$$V = \frac{V_{max} \bullet [S]}{K_m + [S]} + k_{diff} \bullet [S]$$

Kinetic values quoted are the best-fit values ± standard error as derived using Prism 5.0 (Graph Pad Software Inc., San Diego, CA). In this equation V is the observed rate of taurine uptake (nmol/mg protein/min), V_{max} is the maximum rate of taurine uptake, K_m is the taurine affinity binding constant (μM) for the saturable component of taurine uptake, k_{diff} is the rate constant for the non-saturable, diffusion-mediated, taurine influx (ml/mg protein/min) and S is the concentration of extracellular taurine

(μM). From this equation the relative contributions of both the saturable, carrier-mediated, component and the non-saturable, diffusional, component could be determined.

Results

Taurine uptake into SH-SY5Y neuroblastoma cells is regulated by both hyposmolarity and receptor activation. When SH-SY5Y cells were incubated in isotonic buffer (340 mOsM; defined by the osmolarity of the DMEM/fetal calf serum medium in which the cells were grown) that contained 5 μM taurine, the uptake of taurine proceeded linearly for at least 1 h at a rate of 0.067 nmol/mg protein/min (Fig. 3.1A). Taurine uptake was strongly dependent on the concentration of extracellular NaCl (Fig. 3.1B) and was inhibited >95% when incubations were conducted at 4^{o} C (data not shown). Inclusion of 1 mM concentrations of GES, β-alanine and hypotaurine essentially completely blocked taurine uptake (Fig. 3.1C). To the best of our knowledge, the taurine content of SH-SY5Y cells has not previously been reported. HPLC analysis indicated that the taurine content was 57 ± 10 nmol/mg protein (n=7). Given that the intracellular water space in SH-SY5Y cells is 8.5 μl/mg protein (Foster et al., 2008), an intracellular taurine concentration of ~6 mM can be calculated. The concentration of taurine in the growth medium was 12.9 ± 1.1 μM (n=3) and thus taurine is concentrated over 500-fold by SH-SY5Y cells.

Exposure of the cells to hypotonic buffer (230 mOsM; ~30% reduction in osmolarity, a condition previously determined to be optimal for the release of organic osmolytes) resulted in a significant reduction in the rate of taurine influx (0.043 nmol/mg protein/min; $p<0.01$ vs isotonic by ANOVA). Inclusion of the muscarinic cholinergic agonist, Oxo-M (100 μM), resulted in a further attenuation (~50%) of taurine influx (rate = 0.020 nmol/mg protein/min; $p<0.01$ vs. hypotonic

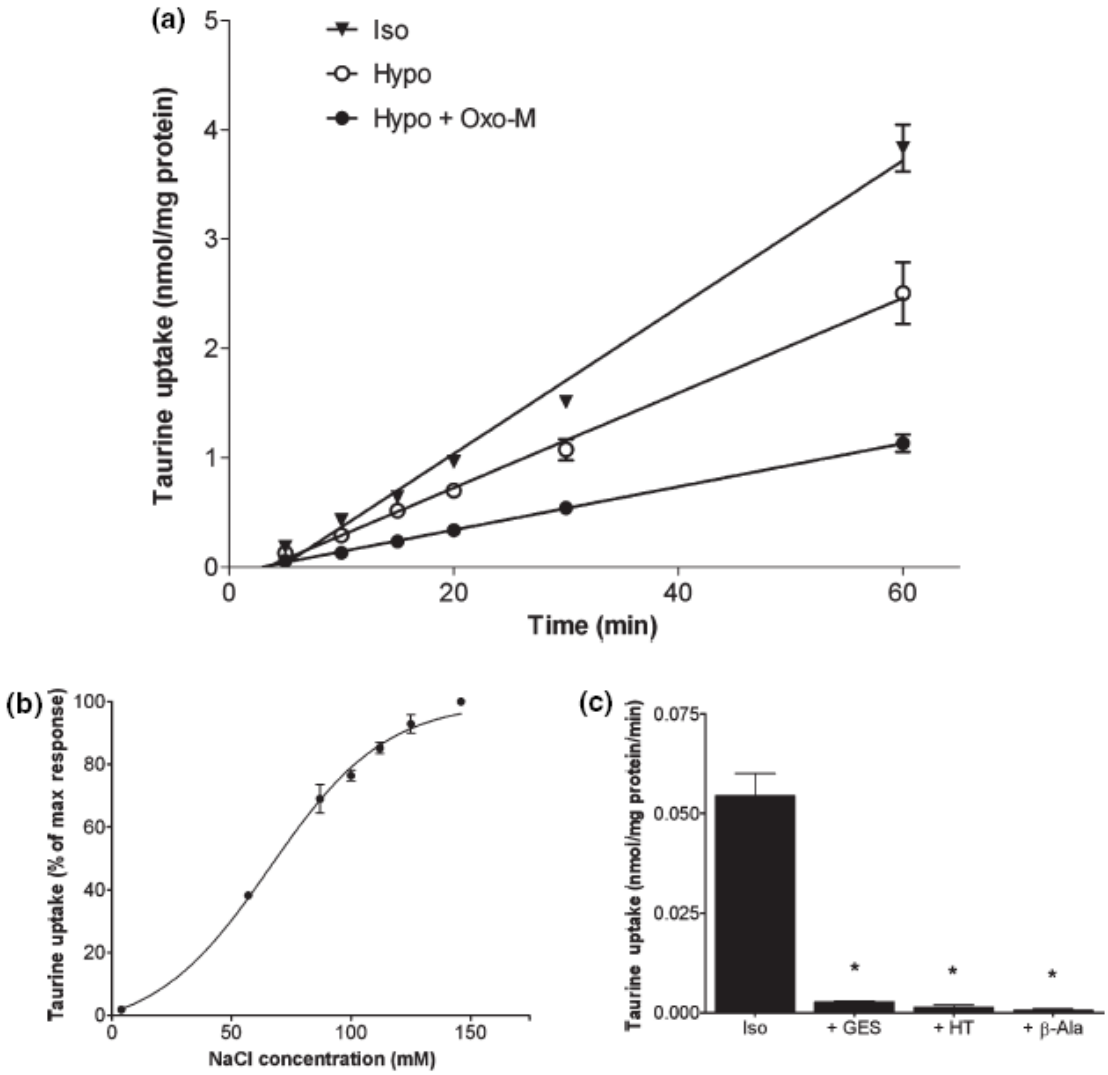

Figure 3.1 Kinetics and characteristics of taurine uptake in SH-SY5Y cells. (A) Cells were incubated in 5 ml of either isotonic (Iso; 340 mOsM; ▼) or hypotonic (Hypo; 230 mOsM) buffer that contained 0.8 μCi ^{3}H-taurine and 5 μM unlabeled taurine in the absence (○) or presence (●) of 100 μM Oxo-M. Reactions were terminated at the times indicated and ^{3}H-taurine uptake was monitored. Results are the means ± SEM of 3 independent experiments, each performed in triplicate. Where error bars are absent the SEM fell within the symbol. Rates of taurine influx were calculated from linear regression analyses of the data. (B) Cells were incubated for 10 min in 5 mL of isotonic buffer containing NaCl at the concentrations indicated (osmolarities were adjusted to 340 mOsM by the addition of sucrose). Results shown are the means ± SEM of 3 independent experiments. Where error bars are absent the SEM fell within the symbol. (C) Taurine uptake was monitored under isotonic conditions (Iso) in the absence or presence of 1 mM concentrations of GES, hypotaurine (HT) or β-alanine (β-Ala). Results are means ± SEM for 3 independent experiments. *, Different from Iso, $p < 0.01$ (by repeated measures ANOVA followed by Dunnett's multiple comparison test).

alone by ANOVA, Fig. 3.1A). The inhibition of taurine uptake observed under conditions of hypotonicity and receptor activation could be detected within 2 min of incubation (the earliest time point examined) and persisted for as long as 1h, a time-frame in which cell volume correction may already be complete. Although taurine influx was linear with time for extended time periods, uptake was routinely monitored after 10 min of incubation to match conditions previously utilized for measurement of osmolyte efflux. The addition of Oxo-M reduced the rate of taurine influx under conditions of hypotonicity in a dose-dependent manner with an EC_{50} of 0.7 μM and a Hill coefficient close to unity (Fig. 3.2). Inclusion of 10 μM atropine, a mAChR antagonist, abolished the ability of Oxo-M to inhibit taurine influx (data not shown).

Activation of multiple GPCRs can attenuate taurine influx. In addition to the mAChR, activation of several other GPCRs has been demonstrated to increase the volume-dependent efflux of both organic and inorganic osmolytes from SH-SY5Y cells, including the protease-activated receptor (PAR) and lysophospholipid receptors (Cheema et al., 2005; Heacock et al., 2006a). To determine whether activation of these receptors also attenuates the uptake of taurine, SH-SY5Y cells were incubated in either isotonic (340 mOsM) or hypotonic (230 mOsM) buffer in the presence or absence of 100 μM Oxo-M, 1.25 nM thrombin (a PAR agonist), or 5 μM sphingosine 1-phosphate (S1P, an agonist at specific lysophospholipid receptors). Although the addition of each of the three agonists resulted in small but significant decreases in the rate of taurine uptake under isotonic conditions, inhibition of taurine

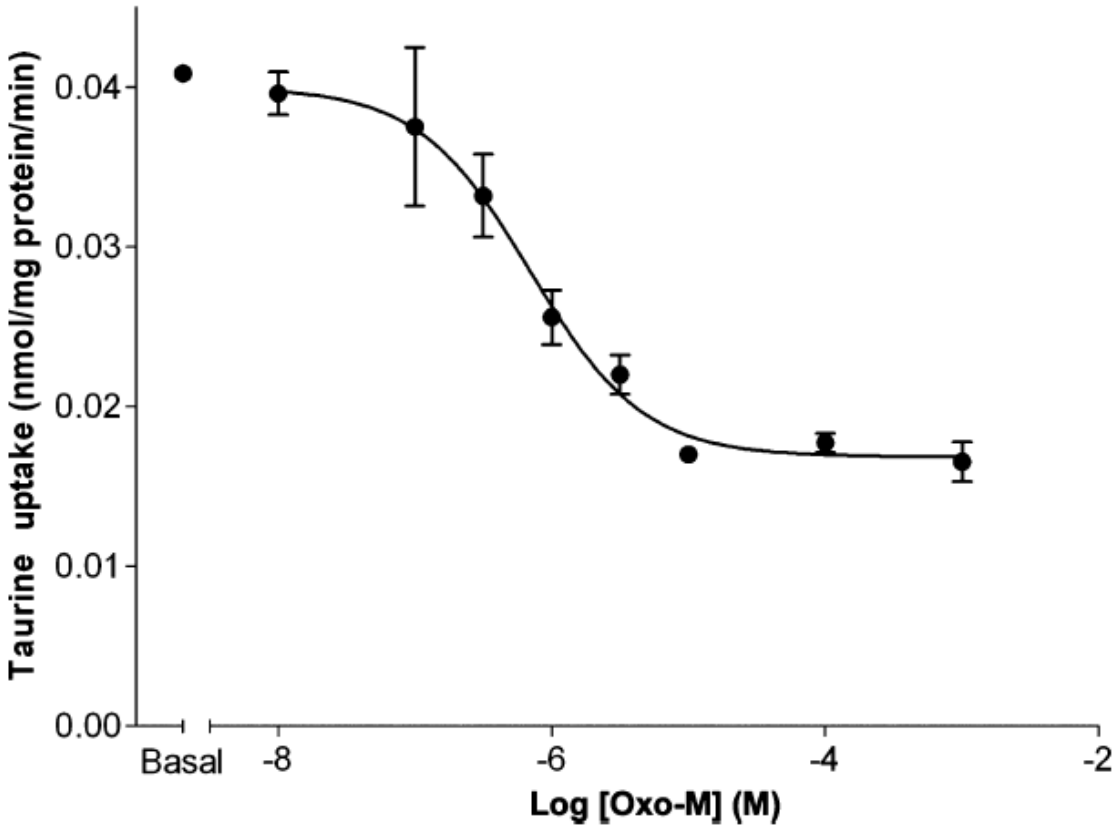

Figure 3.2 Dose-response relationship for Oxo-M-mediated inhibition of taurine influx. SH-SY5Y cells were incubated in 5 mL of hypotonic buffer (230 mOsM) containing 5 μM taurine and Oxo-M at the concentrations indicated. Reactions were terminated after 10 min and taurine uptake monitored. Results are expressed as taurine influx (nmol/mg protein/min) and are the means ± SEM of 3 independent experiments. Where error bars are absent the SEM fell within the symbol. Addition of Oxo-M inhibited taurine influx with an EC_{50} of 0.7 μM and with a Hill coefficient of 1.1.

uptake following receptor activation was more pronounced under hypoosmotic conditions (Fig. 3.3). Thus the *net* ability of Oxo-M to attenuate the rate of taurine uptake was significantly enhanced under hypotonic conditions (0.0113 ± 0.001 vs 0.0141 ± 0.007 nmol/mg protein/min under conditions of isotonicity and hypotonicity, respectively, n=15, $p<0.01$). Similarly, the addition of thrombin resulted in a greater net reduction in taurine uptake under conditions of hypotonicity (0.007 ± 0.002 vs 0.0132 ± 0.002 nmol/mg protein/min, n=5, $p<0.05$). The comparable values for S1P-mediated reductions in taurine uptake were 0.004 ± 0.002 vs 0.011 ± 0.002 nmol/mg protein/min under isotonic and hypotonic conditions, respectively (n=9, $p<0.01$). These results suggest that inhibition of taurine influx is a shared characteristic of GPCRs that have previously been demonstrated to facilitate the volume-dependent efflux of taurine.

Substrate concentration-dependence of taurine uptake in the absence or presence of mAChR activation. Previous studies have demonstrated that, under conditions of hyposmolarity, taurine uptake into neural cells occurs via both saturable (carrier-mediated) and non-saturable (diffusional) components (Schousboe et al., 1976; Olson and Martinho, 2006). To determine the contribution of these two processes to taurine uptake in SH-SY5Y cells, the rate of influx was monitored in the presence of a series of physiologically relevant taurine concentrations, in the absence or presence of 100 μM Oxo-M. Under isotonic conditions, a rectangular hyperbolic dose-response plot was obtained with values of 0.087 ± 0.005 nmol/mg protein/min and 2.5 ± 0.5 μM for the V_{max} and K_m, respectively (Fig. 3.4A). The

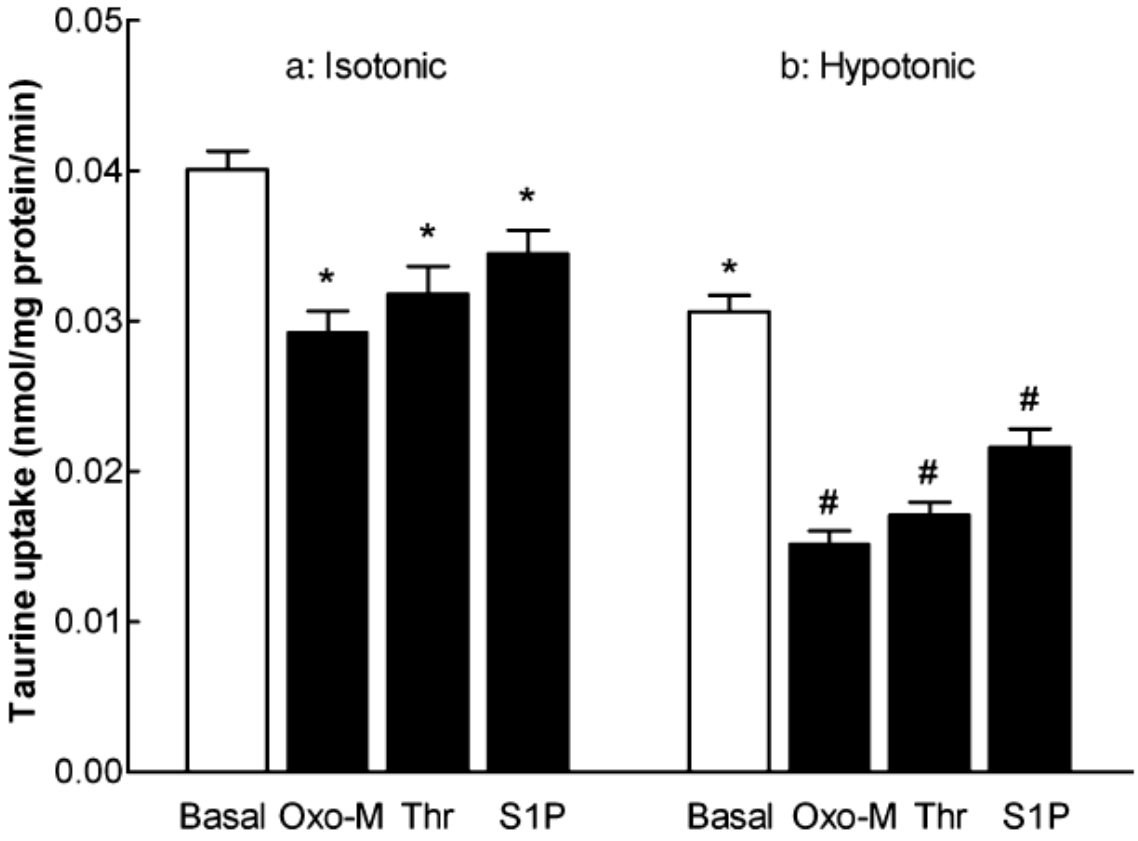

Figure 3.3 Activation of multiple GPCRs can attenuate taurine influx. SH-SY5Y cells were incubated in 5 ml of either isotonic (340 mOsM: A) or hypotonic buffer (230 mOsM: B) in the absence or presence of 100 μM Oxo-M, 1.25 nM thrombin (Thr), or 5 μM S1P. Reactions were terminated after 10 min and taurine uptake monitored. Results are expressed as taurine influx (nmol/mg protein/min) and are the means ± SEM of 15 independent experiments for Oxo-M and 5 or 9 experiments for thrombin or S1P, respectively, each experiment performed in triplicate. *, $p < 0.05$, different from basal release monitored under isotonic (340 mOsM) conditions (by repeated measures ANOVA followed by Dunnett's multiple comparison test). #, $p < 0.05$, different from basal release monitored under hypotonic (230 mOsM) conditions (by repeated measures ANOVA followed by Dunnett's multiple comparison test).

diffusional constant (k_{diff}) was 3.1 ± 6.6 x 10^{-5} ml/mg protein/min. When the cells were incubated in hypotonic buffer, the V_{max} was significantly decreased from that observed under isotonic conditions (0.041 ± 0.002 nmol/mg protein/min; p< 0.001 by ANOVA) whereas the K_m value for taurine uptake was unaltered (2.8 ± 0.5 μM; Fig. 3.4B). The dose-response curve for taurine uptake in the presence of Oxo-M differed significantly from that obtained under conditions of hyposmolarity alone. From the data in Fig. 3.4B, it appeared that inclusion of Oxo-M resulted in an inhibition of taurine uptake at low concentrations (1-20 μM), but that the agonist had little or no effect at higher concentrations of the osmolyte (>50 μM). However, when the data in Fig. 3.4B were fitted to Equation 1 to subtract the diffusional component of taurine influx, rectangular hyperbolic dose-response plots were obtained for Oxo-M-treated cells (Fig. 3.4C). In the presence of Oxo-M, the V_{max} for saturable taurine uptake (0.026 ± 0.003 nmol/mg protein/min) was significantly reduced (~40%: p<0.01, by ANOVA) from control hypotonic incubations, whereas the K_m value for taurine transport did not differ significantly in the presence of Oxo-M (4.7 ± 1.7 μM). However, the diffusional component of taurine uptake was significantly increased (~6-fold) by Oxo-M (2.13 ± 0.40 x 10^{-4} ml/mg protein/min vs. 3.55 ± 2.8 x 10^{-5} ml/mg protein/min in hypotonic buffer alone; p<0.02, by ANOVA). Based on these values, it can be calculated that, even in the presence of Oxo-M, >85% of taurine uptake occurs via the saturable component of transport when monitored in the presence of the standard assay concentration of 5 μM. The diffusional component of taurine influx monitored in the presence of Oxo-M was essentially abolished when DCPIB was included in the assay (k_{diff}=1.57 x 10^{-16}

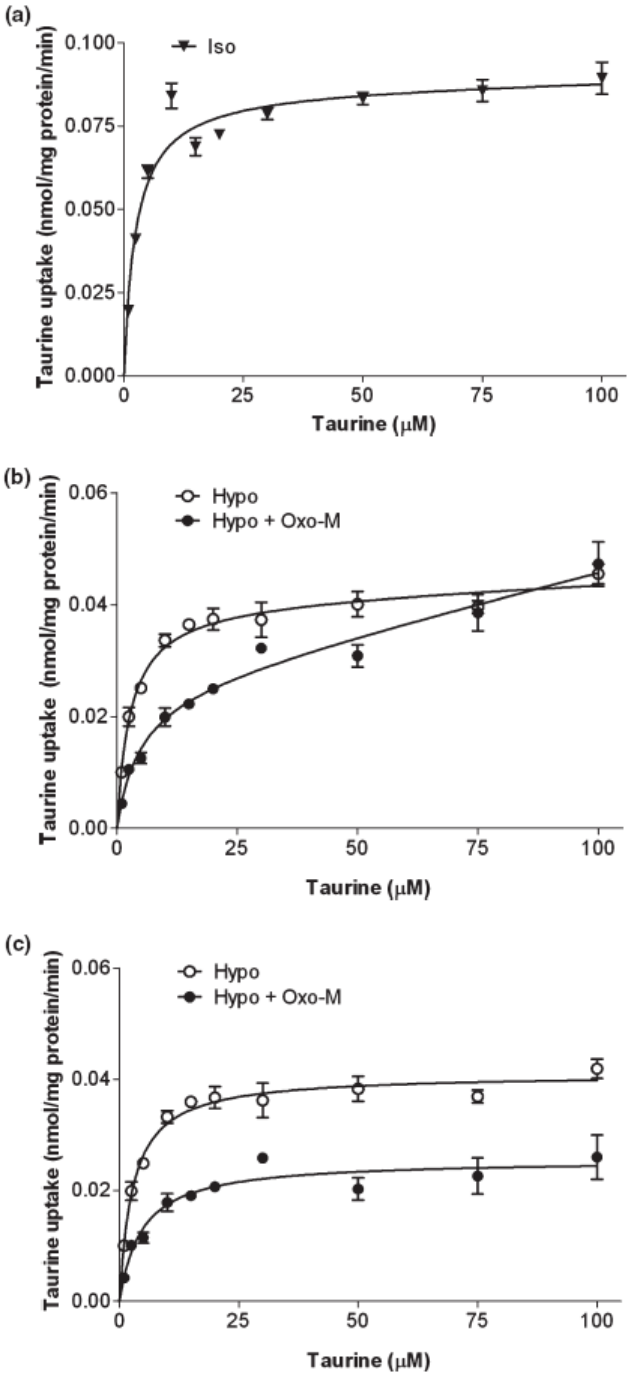

Figure 3.4 Substrate concentration-dependence of taurine uptake. SH-SY5Y cells were incubated in either (A) isotonic buffer A (Iso; 340 mOsM) or (B) hypotonic buffer (Hypo; 230 mOsM) that contained taurine at the concentrations indicated in the absence (open circles) or presence (filled circles) of 100 μM Oxo-M. Taurine uptake was monitored after 10 min and results are the means ± SEM of 3 independent experiments, each performed in triplicate. (C) Plot of taurine influx data obtained from panel B as a function of taurine concentration following subtraction of the non-saturable, diffusion component (see equation 1 in Material and Methods).

ml/mg protein/min). Thus, the non-specific component of taurine uptake appears to be mediated primarily via VSOAC.

Osmolarity dependence of basal- and Oxo-M-attenuated taurine uptake. Activation of mAChRs has previously been demonstrated to facilitate taurine efflux in an osmosensitive fashion by reducing the threshold osmolarity at which osmolyte efflux occurs (Loveday et al., 2003). To assess the effect of osmolarity on taurine uptake, SH-SY5Y cells were incubated in isotonic (340 mOsM), mildy hypertonic (380 mOsM), or hypotonic (290-230 mOsM) buffers under basal- or Oxo-M-stimulated conditions. Two experimental paradigms were employed to evaluate the dependence of taurine uptake on osmolarity. In the first, osmolarities of buffers were adjusted under conditions of a constant NaCl concentration (95 mM) by the addition of sucrose. Because taurine transport is a NaCl-dependent process, this paradigm allows for assessment of the effect of osmolarity *per se* on the rate of taurine influx. Under these conditions, basal uptake was not significantly attenuated until the osmolarity had been reduced by >24% (Fig. 3.5A). Although the addition of Oxo-M induced a significant decrease in the rate of taurine influx under both isotonic and mildly hypertonic conditions (28-38%), the ability of mAChR activation to attenuate taurine influx was significantly increased under conditions of hypotonicity. Inclusion of Oxo-M resulted in a significant reduction in taurine uptake relative to that observed under isotonic conditions when the osmolarity was reduced by ~15% (340 to 290 mOsM; Fig. 3.5A). In the second experimental paradigm, buffers were rendered either hypertonic or hypotonic by increases or decreases, respectively, in

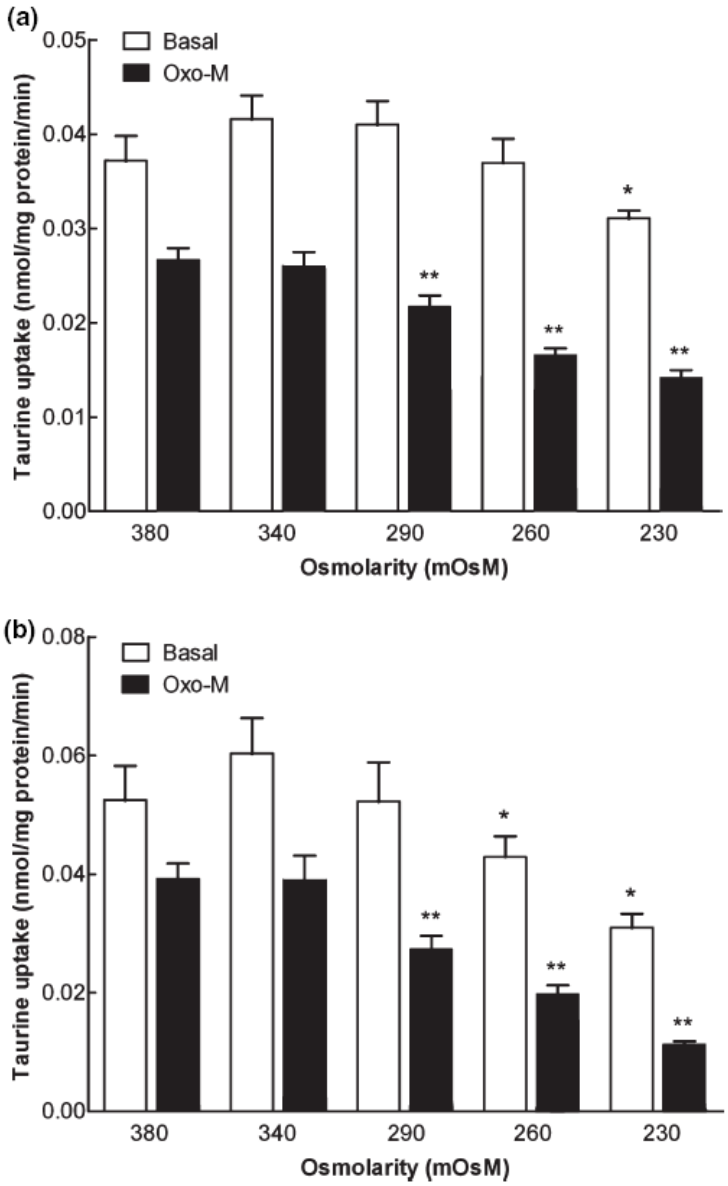

Figure 3.5 Basal- and Oxo-M-stimulated taurine uptake as a function of osmolarity. Taurine influx was monitored during a 10 min incubation in buffer at the osmolarities indicated in the absence (open bars) or presence (closed bars) of 100 *μ*M Oxo-M. (A) Osmolarities of the buffers were adjusted under conditions of a constant NaCl concentration (95 mM). (B) Buffers were rendered hypertonic or hypotonic by an increase or decrease in NaCl concentration, respectively. Results are the means ± SEM of 3-4 independent experiments. *, $p < 0.05$, different from basal release monitored under isotonic conditions (340 mOsM) conditions (by repeated measures ANOVA followed by Dunnett's multiple comparison test). **, $p < 0.05$, different from Oxo-M treatment under isotonic (340 mOsM) conditions (by repeated measures ANOVA followed by Dunnett's multiple comparison test). In both (A) and (B), Oxo-M significantly inhibited taurine uptake at all osmolarities when compared with uptake under basal conditions ($p<0.01$, by paired Student's *t* test).

NaCl concentration (because Na^+ and Cl^- are the primary osmolytes found in plasma and reductions in plasma osmolarity observed under pathological conditions, such as hyponatremia, principally reflect changes in the concentrations of these ions). Under these conditions, the basal uptake of taurine was reduced below that observed under isotonic conditions when the osmolarity was lowered to 260 mOsM, whereas in the presence of Oxo-M, taurine uptake was significantly attenuated following a reduction in osmolarity of 15% (290 mOsM: Fig. 3.5B). Under both experimental paradigms, the ability of Oxo-M to inhibit taurine uptake was most pronounced at 230 mOsM (55-64% relative to basal uptake).

Oxo-M-mediated inhibition of taurine influx: effect of Ca^{2+} depletion and inhibitors of protein kinase C (PKC) activity. The ability of mAChRs to facilitate osmosensitive taurine efflux is dependent upon both the availability of Ca^{2+} and PKC activity. For example, under conditions of a limited Ca^{2+} availability, mAChR-stimulated taurine efflux is inhibited by 60-81%, whereas inhibition of PKC with chelerythrine resulted in a 73% reduction in efflux (Heacock et al., 2006a). mAChR-mediated inhibition of taurine influx was also dependent upon Ca^{2+} availability. Thus although removal of extracellular Ca^{2+} had no effect on the basal influx of taurine under hypotonic (230 mOsM) conditions, it significantly attenuated (~50%) the ability of Oxo-M to inhibit taurine influx. Removal of intracellular Ca^{2+} stores with 1 μM thapsigargin in the absence of extracellular Ca^{2+} elicited a significant increase (~ 30%) in the basal uptake of taurine, but did not further reduce the ability of Oxo-M to inhibit taurine influx (Fig. 3.6). To evaluate a role for PKC in taurine uptake, cells

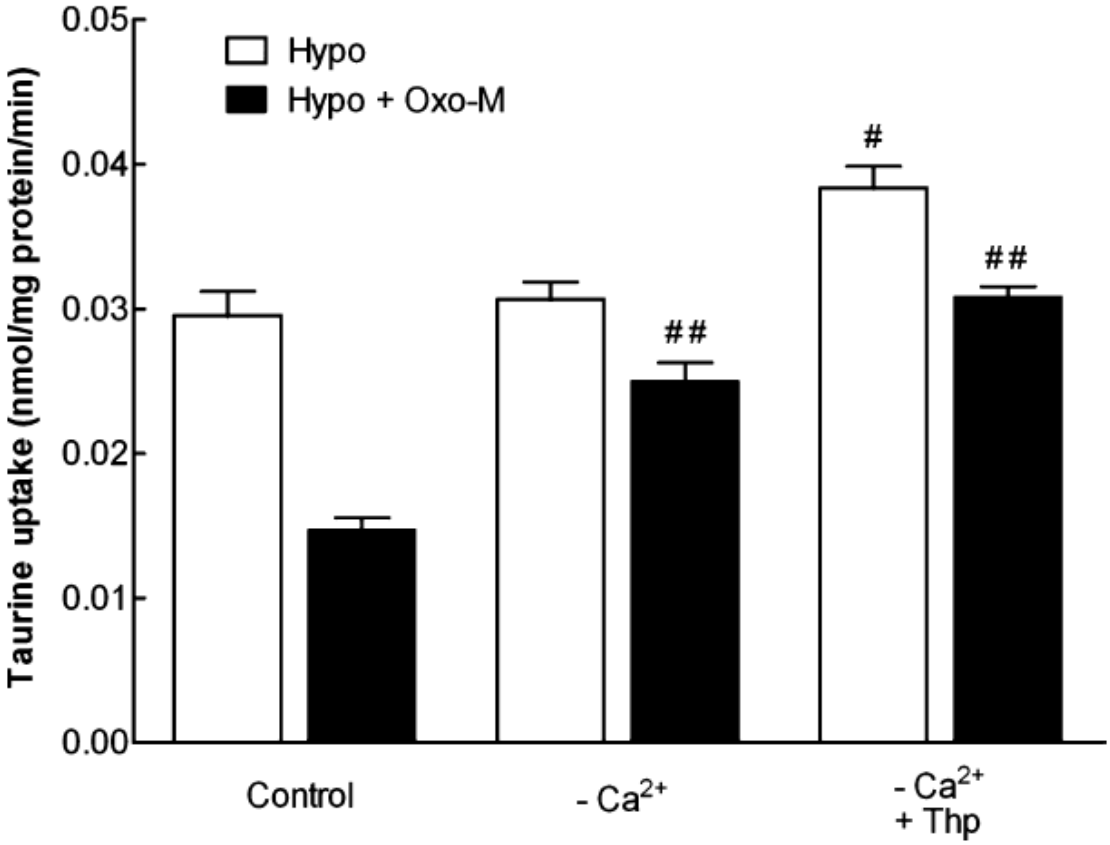

Figure 3.6 The role of extra- and intracellular Ca^{2+} in basal- and Oxo-M-mediated regulation of taurine influx and efflux. SH-SY5Y cells were incubated in the absence (-Ca^{2+}; Ca^{2+} was omitted from buffer and 50 µM EGTA was added) or presence of extracellular Ca^{2+}. For some experiments, cells were pre-incubated for 15 min in isotonic buffer A in the presence of 1 *µ*M thapsigargin (Thp) to deplete intracellular pools of Ca^{2+}. Cells were then incubated in hypotonic buffer (Hypo; 230 mOsM) in the absence (open bars) or presence (closed bars) of 100 *µ*M Oxo-M. Reactions were terminated after 10 min and taurine uptake monitored. Results are expressed as taurine influx (nmol/mg protein/min) and are the means ± SEM of 3-4 independent experiments, each performed in triplicate. #, $p< 0.05$, different from basal taurine uptake under control conditions (by repeated measures ANOVA followed by Dunnett's multiple comparison test). ##, $p< 0.05$, different from taurine uptake monitored in the presence of Oxo-M under basal conditions (by repeated measures ANOVA followed by Dunnett's multiple comparison test).

were pre-incubated for 10 min with 2.5 μM bisindolylmaleimide 1 (BIM), a broad spectrum inhibitor of PKC. BIM had no discernable effect on TauT activity under either isotonic or hypotonic conditions in the absence or presence of Oxo-M (Fig. 3.7A). In contrast, preincubation of the cells with BIM resulted in a 49% reduction in Oxo-M-stimulated taurine efflux (Fig. 3.7B). To further evaluate the possible involvement of PKC in regulation of TauT activity, cells were preincubated with 2.5 μM concentrations of two additional PKC inhibitors, namely Gö 6983 and Ro-31-8220. Although neither of these inhibitors significantly attenuated either basal- or Oxo-M-stimulated taurine influx, both significantly reduced Oxo-M-stimulated (30-53%) taurine efflux (Fig. 3.7C,D). Acute activation of PKC, mediated by the acute addition of 100 nM PMA, did not significantly alter taurine uptake (0.045 ± 0.002 nmol/mg. protein/min vs. 0.050 ± 0.004 nmol/mg. protein/min, n=3).

DCPIB, an inhibitor of VSOAC, attenuates both basal- and Oxo-M-mediated reductions in taurine uptake. Although several pharmacological agents have been found to block the swelling-activated efflux of Cl^- and organic osmolytes from both neural and non-neural cells, the most selective inhibitor identified to date is DCPIB, a derivative of ethacrynic acid (Decher et al., 2001; Best et al., 2004). To determine whether volume-dependent osmolyte efflux and uptake are coupled or independent events, the ability of DCPIB to prevent the inhibition of taurine uptake observed under conditions of hypotonicity and following mAChR activation was evaluated. As previously reported (Heacock et al., 2006a; Cheema et al., 2007), pre-incubation of SH-SY5Y cells with 10 μM DCPIB completely prevented increases in taurine efflux

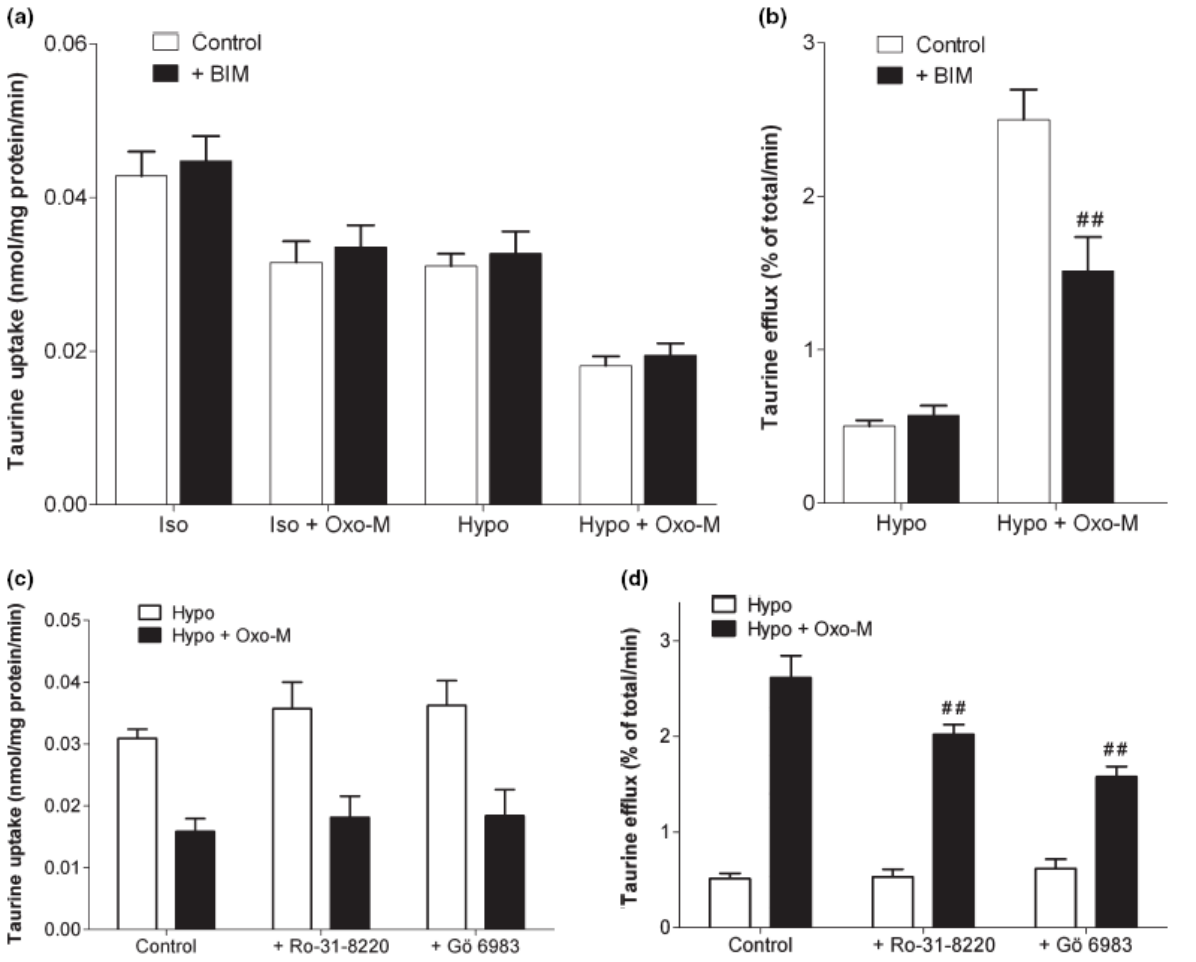

Figure 3.7 The role of PKC in basal- and Oxo-M-mediated regulation of taurine influx and efflux. (A) SH-SY5Y cells were first preincubated, in the presence or absence of 2.5 μM bisindolylmaleimide (BIM), in isotonic buffer for 10 min prior to monitoring taurine uptake under either isotonic (Iso; 340 mOsM) or hypotonic (Hypo; 230 mOsM) conditions in the presence or absence of 100 μM Oxo-M. Reactions were terminated after 10 min and taurine uptake monitored. (B) Cells were treated as described in (A) with the exception that taurine efflux was monitored in cells that had been labeled overnight with [^{3}H]taurine; results are expressed as taurine efflux (percent of total radioactivity initially present in the cells released/min). (C) Conditions were as described in (A) with the exception that cells were preincubated in isotonic buffer in the presence or absence of 2.5 μM Gö 6983 or Ro-31-8220. (D) Taurine efflux was monitored under the conditions described in (C). Results shown are the means ± SEM of 3 independent experiments, each performed in triplicate. ##, $p < 0.01$, different from Hypo + Oxo-M control (by repeated measures ANOVA followed by Dunnett's multiple comparison test).

observed under conditions of hypotonicity alone or following mAChR activation (Fig. 3.8A). Under hypoosmotic conditions, the effects of DCPIB on basal- and Oxo-M-stimulated taurine efflux were dose-dependent with a 3 μM concentration of the inhibitor attenuating ~50% of the responses (Fig. 3.8A). The inhibition of TauT activity observed under basal conditions was also dose-dependently reversed by increases in DCPIB concentration (Fig. 3.8B). In contrast, reversal of Oxo-M-mediated inhibition of TauT activity (i.e. the *net* reduction of uptake due to agonist addition) was not observed until DCPIB concentrations had been increased to >6 μM. Thus, under mAChR-regulated conditions, taurine influx is not attenuated by DCPIB until efflux is substantially inhibited. DCPIB had no effect on basal taurine uptake under isotonic conditions (0.040 ± 0.002 vs 0.044 ± 0.001 nmol/mg protein/min in the absence or presence of 10 μM DCPIB, respectively, n=5), a result that indicates the absence of any direct effect of DCPIB on the taurine transporter itself. We then extended the studies with DCPIB to include measurement of K^+ fluxes (as monitored by $^{86}Rb^+$) since we have recently reported that hypotonicity and mAChR activation can regulate both the influx and efflux of the cation in these cells (Foster et al., 2008). Although a reduction in osmolarity of ~30% resulted in little or no change in either K^+ efflux or influx, inclusion of Oxo-M resulted in significant increases in both parameters. Inclusion of DCPIB inhibited the mAChR-mediated increases in both $^{86}Rb^+$ efflux and influx by >80% but had little or no effect on K^+ fluxes monitored under isotonic conditions (Fig. 3.9). These results suggest that, in SH-SY5Y cells, volume-dependent osmolyte efflux mediated via VSOAC may result in a coordinated regulation of the uptake of both organic and inorganic osmolytes.

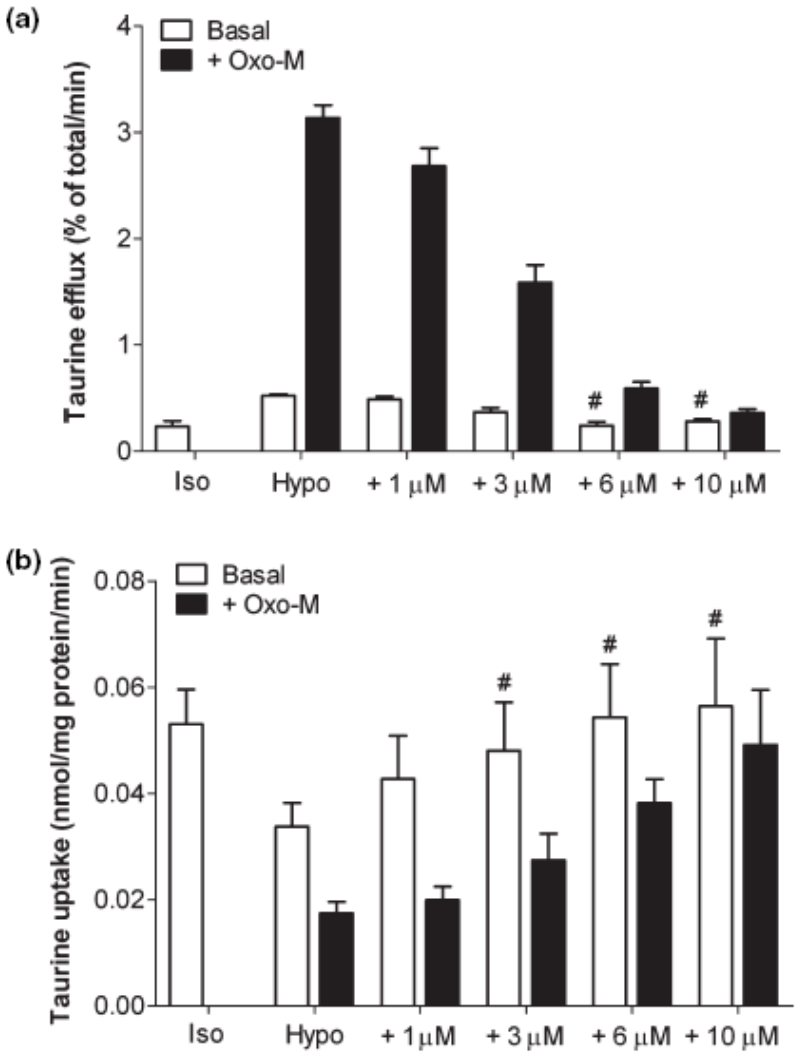

Figure 3.8 DCPIB attenuates both basal- and Oxo-M-mediated changes in taurine efflux and uptake. (A) SH-SY5Y cells that had been labeled overnight with [^{3}H]taurine were incubated in either isotonic (Iso; 340 mOsM) or hypotonic (Hypo; 230 mOsM) buffer in either the absence or presence of 100 μM Oxo-M (solid bars). To determine the effect of DCPIB, cells were pre-incubated for 10 min in isotonic buffer in the presence of DCPIB at the concentrations indicated prior to the assay. The buffers were then aspirated and replaced with fresh hypotonic buffer that also contained DCPIB at the concentrations used during the preincubation. (B) Cells were treated as described in (A) with the exception that [^{3}H]taurine uptake into unlabeled cells was monitored. For both efflux and uptake measurements, reactions were terminated after 10 min and results are the means ± S.E.M. of 3-4 independent experiments, each performed in triplicate. #, $p < 0.05$, different from Hypo basal (by repeated measures ANOVA followed by Dunnett's multiple comparison test). At all concentrations of DCPIB, with the exception of 10 μM, Oxo-M addition significantly increased taurine efflux or attenuated taurine uptake when compared to basal conditions ($p<0.05$, by paired Student's *t* test).

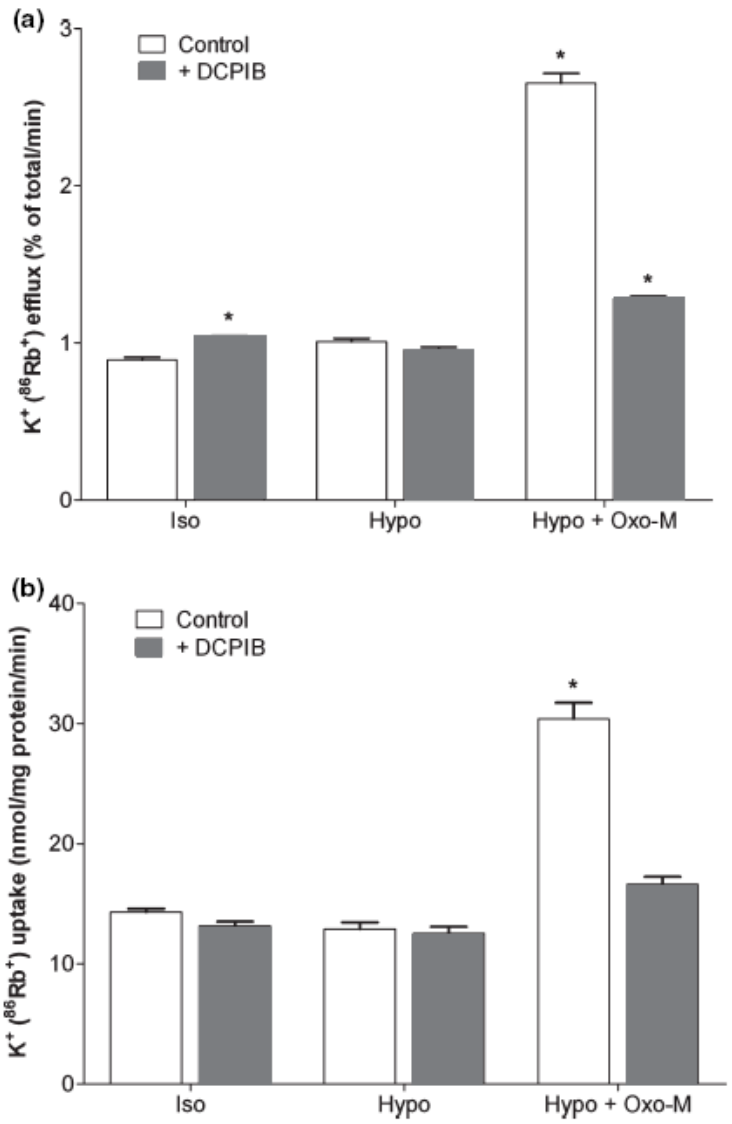

Figure 3.9 DCPIB attenuates both basal- and Oxo-M-mediated changes in $^{86}Rb^+$ efflux and uptake. (A) SH-SY5Y cells that had been labeled overnight with $^{86}Rb^+$ were washed twice with isotonic buffer and then incubated in isotonic (Iso; 340 mOsM) or hypotonic (Hypo; 230 mOsM) buffer in either the absence or presence of 100 μM Oxo-M. To determine the effect of DCPIB (shaded bars), cells were pre-incubated for 10 min in isotonic buffer in the presence of 10 μM DCPIB prior to the assay. The media were then aspirated and replaced with hypotonic buffer that also contained 10 μM DCPIB. (B) Cells were treated as described in (A) with the exception that $^{86}Rb^+$ uptake into unlabeled cells was monitored. For both efflux and uptake measurements, reactions were terminated after 10 min and results expressed as either $^{86}Rb^+$ efflux (percent of total radioactivity initially present in the cells released/min) or, alternatively as $^{86}Rb^+$ influx (nmol/mg protein/min) and are the means ± S.E.M. of 4-5 independent experiments, each performed in triplicate. *, $p < 0.05$, different from Iso (by repeated measures ANOVA followed by Dunnett's multiple comparison test).

Discussion

Although the release of the quantitatively major osmolyte, taurine, can be enhanced from a variety of neural preparations in response to both hyposmolarity and the activation of specific GPCRs, the possibility that the re-uptake of taurine is subject to similar regulation has received little attention. This issue is of potential physiological significance since the net loss of an osmolyte from a tissue will reflect not only changes in its efflux but also in its re-uptake. The major finding to emanate from the present study is that, in SH-SY5Y neuroblastoma cells, the uptake of taurine is inhibited by both a reduction in osmolarity and in response to activation of those GPCRs (mAChR, S1P and PAR) that have previously been demonstrated to facilitate volume-dependent osmolyte efflux. Thus hyposmolarity and activation of GPCRs serve not only to enhance the efflux of taurine from SH-SY5Y cells but also to limit its re-uptake, the net effect being a more pronounced loss of the osmolyte.

Under isotonic conditions, taurine uptake into SH-SY5Y cells is mediated via a high–affinity, Na^+-dependent, saturable transport system with pharmacological characteristics shared by the two cloned taurine transporters TauT-1 and TauT-2 (Liu et al., 1992; Smith et al., 1992; Pow et al., 2002). The K_m for taurine uptake in SH-SY5Y cells (2.5 μM), is similar to that reported for TauT-2 (Liu et al., 1992; Pow et al., 2002), an isoform of TauT that is widely distributed across different brain regions. In contrast, a lower affinity for uptake has been reported for the cloned TauT-1 transporter or for taurine uptake into astrocyte preparations (Km=20-45 uM; Holopainen et al., 1987; Sanchez-Olea et al., 1991; Smith et al., 1992). The

calculated intracellular taurine concentration in SH-SY5Y cells (6-7 mM) is similar to that reported for whole brain (McIlwain and Bachelard, 1971), but lower than that observed for astrocytes (Beetsch and Olson, 1993; Olson, 1999).

Exposure of SH-SY5Y cells to hypotonicity resulted in a sustained inhibition of taurine uptake, that was attributable to a reduction in the V_{max} for transport whereas the K_m value was unaffected. This reduction in taurine uptake, which could be observed under conditions in which the Na^+ concentration of the buffer was either lowered or maintained constant, was evident when the osmolarity was reduced by >20%. Under the hypotonic conditions routinely employed (~30% reduction in osmolarity), >99% of taurine uptake monitored at a 5 μM concentration was mediated via the high-affinity transport system. The diffusional component of taurine uptake in SH-SY5Y cells was minimal under isotonic conditions and little or no increase in this parameter occurred under hypoosmotic conditions (Fig. 3.4). Our observation that hypotonicity reduces the V_{max} for taurine uptake in SH-SY5Y cells without an effect on the K_m is in agreement with the results of a recent study of primary hippocampal neurons in which taurine uptake was also attenuated (by 48%) in response to a 30% reduction in osmolarity (Olson and Martinho, 2006). Although the mechanism underlying the reduction of TauT activity under basal hypoosmotic conditions remains to be determined, possibilities to be considered include the involvement of a tyrosine kinase and internalization of the transporter (Han et al., 2006; Olson and Martinho, 2006).

Activation of GPCRs also resulted in an attenuation of taurine uptake in SH-SY5Y cells. Following activation of the mAChR, PAR-1 or S1P receptors, a small

attenuation of taurine uptake was observed under isotonic conditions but the extent of receptor-mediated inhibition was enhanced when the cells were incubated in a hypoosmotic buffer (Fig. 3.3). To the best of our knowledge, this is the first report of receptor-mediated, volume-dependent, regulation of the uptake of an organic osmolyte. Previously, we have demonstrated that the influx of K^+ is also under receptor control in these cells (Foster et al., 2008). Attenuation of taurine uptake in SH-SY5Y cells under hypoosmotic conditions was examined in more detail for the mAChR. The addition of Oxo-M resulted in a concentration-dependent inhibition of taurine uptake with an EC_{50} of 0.7 μM, whereas the comparable value for Oxo-M-stimulated taurine efflux is 2.3 μM (Heacock et al., 2004). Receptor-mediated inhibition of taurine uptake under hypoosmotic conditions was sustained for at least 1 h of incubation and could be observed under conditions of relatively limited reductions in osmolarity (15%: Fig. 3.5). mAChR-mediated reductions in taurine uptake under hypoosmotic conditions were primarily due to a reduction in V_{max}, with little or no effect on the K_m (Fig. 3.4C).

A common characteristic of those GPCRs that have been previously demonstrated to facilitate the efflux of osmolytes in SH-SY5Y cells is a dependence on both Ca^{2+} availability and PKC activity. Similarly, the magnitude of the mAChR-mediated reduction in taurine influx was also partially reversed (50-60%) following removal of extracellular Ca^{2+}, although the dependence on Ca^{2+} was less marked than that previously observed for stimulated taurine efflux (Cheema et al., 2007). However, mAChR-mediated changes in taurine efflux and influx appeared to differ significantly in their dependence upon PKC activity. Thus, whereas inclusion of BIM,

Gö 6983 or Ro-31-8220, three broad-spectrum inhibitors of PKC, had no effect on either basal-or receptor-mediated changes in taurine influx, all three inhibitors significantly attenuated mAChR-stimulated taurine efflux (~30-53%: Fig. 3.7). Two interpretations of these results are possible. The first is that mAChR activation independently regulates taurine influx and efflux and that these processes are differentially sensitive to PKC inhibition. Alternatively, mAChR-mediated changes in VSOAC activity may be a prerequisite for the regulation of taurine influx and a relatively small increase in osmolyte efflux is sufficient for maximal attenuation of TauT activity. This possibility is further discussed below in the context of the results obtained with DCPIB.

Changes in the magnitude of taurine influx and efflux that occur under basal- and mAChR-stimulated conditions are not strictly proportional. For example, TauT activity is inhibited to a similar extent following either a 30% reduction in osmolarity or activation of mAChRs under hypoosmotic conditions. In contrast, much larger increases in taurine efflux are elicited in response to the addition of Oxo-M than to a reduction in osmolarity (see Fig. 3.8). However, evidence to suggest that basal- and receptor-mediated changes in the influx and efflux of taurine are linked, rather than independent, events was obtained from experiments with DCPIB, a selective inhibitor of VSOAC. Inclusion of a maximally effective concentration of DCPIB (10 μM) resulted not only in a >70-90% inhibition of basal-and mAChR-mediated increases in taurine efflux, as would be predicted, but the inhibitor also prevented the attenuation of taurine influx under these conditions. Since DCPIB has no direct effect on taurine transporter activity, the most parsimonious interpretation of these

results is that the activation of VSOAC and regulation of TauT activity are mechanistically inter-related events. Reversal of changes in basal taurine efflux and influx by DCPIB demonstrated a similar dose-dependence. In contrast, mAChR-mediated increases in taurine efflux were more sensitive to lower concentrations of DCPIB than was the attenuation of taurine influx (Fig. 3.8). This result, suggests that for maximal attenuation of taurine influx to occur, only relatively limited increases in VSOAC activity are required and that more substantial increases in the rate of efflux do not result in further reductions of taurine influx. This observation may be relevant when considering the lack of PKC regulation of mAChR-attenuated taurine influx, since none of the inhibitors employed blocked efflux by >53%. Further evidence to suggest that VSOAC activity may be linked to osmolyte influx was obtained from experiments in which DCPIB also prevented mAChR-mediated changes in the efflux and influx of K^+ (Fig. 3.9). Although a mechanistic link has yet to be established, the results raise the possibility that the activation of VSOAC is a prerequisite for regulatory changes in the release and uptake of both organic and inorganic osmolytes.

Under conditions of chronic hyponatremia the brain selectively retains inorganic osmolytes whereas organic osmolytes are lost (Pasantes-Morales et al., 2002b; Massieu et al., 2004). Hyposmolarity has been demonstrated to result in an increase in a Ca^{2+}-dependent exocytotic release of neurotransmitters from nerve-ending preparations (Tuz et al., 2004; Tuz and Pasantes-Morales, 2005). These neurotransmitters may then regulate the activities of GPCRs, present on neighboring cells, that are linked to volume regulation. Our current and previous studies in which

we demonstrate that activation of GPCRs can regulate not only the efflux but also the influx of both organic and inorganic osmolytes may provide, in part at least, an explanation for the differential retention *in vivo* of organic and inorganic osmolytes observed during hyponatremia. There is an increasing body of evidence to suggest that GPCRs can regulate the efflux of osmolytes from a variety of tissues (Fisher et al., 2008; Franco et al., 2008; Vazquez-Juarez et al., 2008a). The observation that GPCRs can also regulate the reuptake of osmolytes further emphasizes the potential importance of this class of receptors in osmoregulation.

Chapter 4

Muscarinic Receptor Stimulation of D-Aspartate Uptake into Human SH-SY5Y Neuroblastoma Cells is Attenuated by Hyposmolarity

Summary

In addition to its function as an excitatory neurotransmitter, glutamate also plays a major role as an osmolyte within the central nervous system (CNS). Accordingly, mechanisms that regulate glutamate release and uptake are of physiological importance not only during conditions in which cell volume remains constant, but also when cells are subjected to hypoosmotic stress. In the present study, the ability of muscarinic cholinergic receptors (mAChRs) to regulate the uptake of glutamate (monitored as D-aspartate) into human SH-SY5Y neuroblastoma cells under isotonic or hypotonic conditions has been examined. In isotonic media, agonist activation of mAChRs resulted in a significant increase (250-300% of control) in the uptake of D-aspartate and concurrently, a cellular redistribution of the excitatory amino acid transporter 3 (EAAT3) to the plasma membrane. mAChR-mediated increases in D-aspartate uptake were potently blocked by the EAAT3 inhibitor, L-β-threo-benzyl-aspartate. In hypotonic media, the

ability of mAChR activation to facilitate D-aspartate uptake was significantly attenuated (40-50%) and the cellular distribution of EAAT3 was disrupted. Reduction of mAChR-stimulated D-aspartate uptake under hypoosmotic conditions could be fully reversed upon re-exposure of the cells to isotonic media. Under both isotonic and hypotonic conditions, mAChR-mediated increases in D-aspartate uptake were dependent upon cytoskeletal integrity, protein kinase C (PKC) and phosphatidylinositol 3-kinase (PI3K) activities and the availability of intracellular Ca^{2+}. In contrast, a dependence on extracellular Ca^{2+} was only observed under isotonic conditions. The results suggest that although the uptake of D-aspartate into SH-SY5Y cells is enhanced following mAChR activation, this process is markedly attenuated by hyposmolarity.

Introduction

The maintenance of cell volume is an ancient homeostatic mechanism that is of prime importance to the CNS due to the restrictions imposed by the skull and the need to maintain correct spatial relationships between cells to avoid changes in neuronal excitability (Sykova, 2004). Cell swelling can occur under physiological conditions (following intense neuronal discharge) or result from pathological causes, such as a reduction in plasma osmolarity (hypoosmotic swelling). While the renal system normally maintains plasma osmolarity within a very narrow range, this regulation can be compromised under pathological conditions, the most prevalent of which is hyponatremia, the most commonly diagnosed electrolyte disorder in clinical practice. Hyponatremia is defined as a reduction in the serum Na^+ concentration from 145 mEq/L to ≤ 136 mEq/L with severe cases resulting in up to 25% reductions in the concentration of serum Na^+. Changes in brain cell volume (<5%) can have profound effects within the CNS and lead to neurological symptoms such as nausea, headache, coma and ultimately, respiratory arrest (Bhardwaj, 2006; Lien and Shapiro, 2007). Under hypoosmotic conditions, cells initially swell and then correct their volume through the release of both inorganic and organic osmolytes, a process that results in the exit of obligated water. Glutamate, along with taurine and myo-inositol represent the major organic osmolytes that, together with the inorganic osmolytes (K^+ and Cl^-), account for the bulk of the osmolytes used by neural cells. In response to a reduction in osmolarity most cells appear to extrude organic osmolytes and Cl^- through a common volume-sensitive organic osmolyte and anion

channel whereas the release of K^+ occurs via one or more K^+ channels. Although volume control has previously been largely considered to be an intrinsic property of the cell, there is now abundant evidence to suggest that volume-dependent osmolyte release from a variety of neural- and non-neural preparations can be enhanced following the activation of a wide spectrum of G-protein-coupled receptors (GPCRs; for reviews see Fisher et al., 2008; Franco et al., 2008; Vazquez-Juarez et al., 2008a). Receptor activation appears to not only enhance the magnitude of osmolyte release but also to lower the osmotic threshold at which release occurs, thereby facilitating the ability of cells to respond to small, physiologically relevant, reductions in osmolarity.

Although the net loss of an osmolyte from a cell maintained under hypoosmotic conditions is dependent upon both its release and subsequent influx, the possibility that activation of GPCRs may regulate not only the efflux, but also the re-uptake of osmolytes, has received little attention. In this context, we have recently demonstrated that the activation of mAChRs present on human SH-SY5Y neuroblastoma cells results in not only a facilitation of the volume-dependent efflux of both taurine and K^+, but also a regulation of the re-uptake of both osmolytes. However, whereas mAChR activation results in an *increase* in the uptake of K^+, and thereby its retention in cells, a *decrease* in the influx of taurine is observed, an effect that promotes the loss of this organic osmolyte from cells (Foster et al., 2008; Foster et al., 2009). Although an increase in the extracellular concentration of a relatively inert compound such as taurine might have minimal cellular consequences, a similar increase in the concentration of extracellular glutamate, a quantitatively important

organic osmolyte in the CNS (Fisher et al., 2008), would be potentially deleterious due to its excitotoxic effects. Accordingly, in the present study we have evaluated the ability of osmolarity to regulate the uptake of glutamate into SH-SY5Y neuroblastoma, a model neuronal cell line. In contrast to our previous results obtained for taurine influx, we find that under isotonic conditions, glutamate uptake (monitored as D-aspartate) is significantly enhanced following mAChR activation. This effect appears to be primarily mediated by a cellular redistribution of EAAT3, a neuronal-specific transporter, to the plasma membrane. The increase in D-aspartate uptake was dependent upon the availability of extra- and intracellular Ca^{2+}, PKC and PI3K activities and the integrity of the cytoskeleton. Incubation of the cells under hypoosmotic conditions significantly attenuated mAChR-mediated D-aspartate uptake, an effect that could be reversed upon re-exposure of the cells to an isotonic medium. Although receptor-mediated increases in D-aspartate uptake monitored under hypoosmotic conditions were also dependent upon the activities of PKC and PI3K and cytoskeletal integrity, there was little or no dependence on extracellular Ca^{2+}. The results suggest that hyposmolarity significantly attenuates the ability of mAChRs to facilitate glutamate accumulation in SH-SY5Y cells and provides further evidence for a role for GPCR activation in the regulation of osmolyte influx.

Materials and Methods

Materials. D-[2,3-^{3}H]Aspartic acid (23.0 Ci / mmol) and myo-[2-^{3}H]inositol (15.0 Ci / mmol) were obtained from GE Healthcare (Chalfont St. Giles, UK). [^{3}H]Quinuclidinyl benzilate (QNB: 50.5 Ci / mmol) was obtained from Perkin Elmer Life and Analytical Sciences (Waltham, MA). Oxotremorine-M, sphingosine 1-phosphate, thrombin, atropine, D-aspartic acid, wortmannin, colchicine, and L-β-*threo*-benzyl-aspartate were purchased from Sigma-Aldrich (St. Louis, MO). DL-*threo*-β-benzyloxyaspartic acid, dihydrokainic acid and L-(-)-*threo*-3-hydroxyaspartic acid were obtained from Tocris Bioscience (Ellisville, MO). Thapsigargin, ionomycin, phorbol-12-myristate-13 acetate, cytochalasin D and bisindolylmaleimide 1 were obtained from Calbiochem (San Diego, CA). Lysophosphatidic acid was purchased from Avanti (Alabaster, AL). Dulbecco's Minimal Essential Medium (DMEM, low glucose) and 50x penicillin / streptomycin were obtained from Invitrogen (Carlsbad, CA). Fetal calf serum was obtained from Lonza Walkersville Inc. (Walkersville, MD). Tissue culture supplies were obtained from Corning Inc. (Corning, NY), Starstedt (Newton, NC) and BD BioSciences (San Jose, CA). Universol was obtained from MP Biomedicals (Solon, OH).

Antibodies. Rabbit anti-Rat EAAC1 affinity pure IgG (EAAC11-A, recognizes the human homolog, EAAT3) was obtained from Alpha Diagnostic International (San Antonio, TX) and used at a 1:1,000 dilution. Goat anti-early endosomal antigen (anti-EEA1) affinity purified IgG (sc-6415; used at a 1:1,000 dilution), Goat anti-actin

HRP-conjugated polyclonal IgG (sc-1616-HRP; used at a 1:10,000 dilution), Goat anti-Rabbit HRP-conjugated IgG (sc-2004; used at a 1:10,000 dilution), and Donkey anti-Goat HRP-conjugated IgG (sc-2033; used at a 1:12,500 dilution) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The following primary antibodies were not able to detect EAAT1 or EAAT2 in either SH-SY5Y cell lysate or subcellular fractions: Guinea Pig anti-EAAT1 (Ab1782, Millipore; Billerica, MA), Guinea Pig anti-EAAT2 (Ab1783, Millipore), Goat anti-EAAT1 (sc-7758, Santa Cruz) and Rabbit anti-EAAT1 (sc-15136, Santa Cruz).

Cell culture conditions. Human SH-SY5Y neuroblastoma cells (passages 70-89) or C6 glioma cells (passages 46-48) were grown in tissue culture flasks (75 cm^2 / 250ml) in 20 ml of DMEM supplemented with 10% (v/v) of fetal calf serum and 1% penicillin / streptomycin with the osmolarity of the media adjusted to 290 mOsM. Cells were grown at 37^oC in a humidified atmosphere containing 10% CO_2. The medium was aspirated and cells detached from the flask with a trypsin-versene mixture (Lonza Walkersville Inc; Walkersville, MD). Cells were then resuspended in DMEM / 10% fetal calf serum with penicillin / streptomycin (290 mOsM) and subcultured into 35-mm, six-well culture plates at a density of 200 to 300,000 cells / well for 4 to 5 days. Cells that had reached 70-90% confluence with a protein content of ~0.25 mg protein / well were routinely used.

Measurement of D-aspartate uptake. SH-SY5Y cells were washed once with 2 ml of isotonic Buffer A (116 mM NaCl, 5.6 mM KCl, 2.2 mM $CaCl_2$, 3.6 mM $NaHCO_3$, 1

mM $MgCl_2$, 30 mM HEPES, pH 7.4, and 1 mg / ml D-glucose, ~290 mOsM) prior to assay. Cells were routinely incubated (unless stated otherwise) in 5 ml of hypotonic buffer A (230 mOsM; 90 mM NaCl) or isotonic buffer A (290 mOsM; adjusted by the addition of 60 mM sucrose to hypotonic buffer A to maintain a constant NaCl concentration) containing 10 μM D-[^{3}H]aspartate (0.3 μCi / ml: used as a marker for L-glutamate) in the presence or absence of agonist at 37^{o}C. The 5 ml assay volume (50 nmol of added D-aspartate) was chosen to minimize any dilution of specific activity caused by the release of intracellular glutamate during the uptake assay under hypotonic conditions according to the following calculations. In SH-SY5Y cells, the concentration of L-glutamate is 100 nmol / mg of protein (see Results). During a 10 min incubation period in the presence of Oxo-M under conditions of a 20% reduction in osmolarity, 5-10% of D-aspartate is released (Heacock et al., 2004). Assuming that D-aspartate release reflects that of L-glutamate, it can be calculated that ~ 2 nmol of L-glutamate would be released, which would contribute < 5% to the extracellular pool.

At the times indicated, the extracellular medium was aspirated and cells were immediately washed with 2 ml of isotonic buffer A prior to lysis in 2 ml of 0.1 M NaOH. Aliquots of lysate (1 ml) were removed and radioactivity determined after the addition of 7.5 ml Universol scintillation fluid and 50 μl of glacial acetic acid (to quench chemiluminescence). In all measurements, radioactivity associated with the cell monolayer at the zero time point was subtracted from the observed value. Protein contents of cell lysates were determined using a bicinchoninic acid protein assay reagent kit (Pierce, Rockford, IL). Rates of D-aspartate uptake into SH-SY5Y

cells were calculated as pmol / mg protein / min. Throughout the study, 'basal' uptake of D-aspartate is defined as that which occurs at a specified osmolarity in the absence of an agonist. Since measurement of D-aspartate uptake in the presence of Oxo-M represents the sum of both the basal- and agonist-stimulated components, percentage changes in the rates of Oxo-M-stimulated D-aspartate uptake reported in this study are calculated on the basis of the *net* changes in the agonist mediated component of uptake, i.e. D-aspartate uptake observed in the presence of Oxo-M minus the basal uptake observed under the same experimental conditions.

Glutamate mass measurements. SH-SY5Y cells were washed once with 2 ml of isotonic buffer A and detached by the addition of 1 ml of Puck's D1 solution (Heacock et al., 2004). Six wells of detached cells were pooled, centrifuged (5 min at 1,750 x g) and the resulting supernatant was aspirated. Cell pellets were lysed by the addition of 1 ml of boiling dH_2O for 10 min with intermittent sonication. Intracellular glutamate was measured via a spectrophotometric method (Lund and Bergmeyer, 1986) by means of a glutamate assay kit (Sigma Aldrich; St. Louis, MO). Results are expressed as nmol endogenous glutamate / mg protein.

Measurement of Phosphoinositide Turnover. To monitor phosphoinositide turnover, SH-SY5Y cells that had been prelabeled with 10 μCi / mL [^{3}H]inositol for 96 hrs were washed with isotonic buffer A and then incubated in buffer of the osmolarity indicated containing 5 mM LiCl in the presence or absence of 100 μM Oxo-M. The

accumulation of radiolabeled inositol phosphates present in the trichloroacetic acid cell lysates was determined as described previously (Thompson and Fisher, 1990).

Subcellular Fractionation. A modified procedure of the method of Stadel et al. (1983) was used essentially as previously reported (Sorensen et al., 1997). Twenty flasks of SY5Y cells (5 flasks / treatment) were washed with 5 ml of isotonic buffer A at 37 ^{o}C prior to treatment in either hypotonic (230 mOsM; 90 mM NaCl) or isotonic buffer A (290 mOsM; rendered isotonic by the addition of 60 mM sucrose to hypotonic buffer) in the absence or presence of Oxo-M for 10 min. Reactions were terminated by aspiration of the media and the addition of 5 ml of ice-cold isotonic buffer A to the flasks that were maintained on ice. The buffer was then aspirated and cells detached by the addition of 5 ml of ice-cold Puck's D1 Solution (Heacock et al., 2004). Cells were then centrifuged at 1,750 x g for 10 min at 4^{o}C and the supernatants aspirated. Cell pellets were lysed by gentle homogenization in 10 mL TE buffer (10 mM Tris-HCl, pH 7.4; 2 mM EDTA) containing complete protease inhibitors (Roche Diagnostics, Indianapolis, IN). Cell lysates were then centrifuged at 1,000 x g for 10 min at 4^{o}C to obtain a crude nuclear fraction (N_1). The N_1 fraction was re-suspended in 10 ml TE buffer containing protease inhibitors, re-homogenized and centrifuged at 1,000 x g for 10 min at 4^{o}C. Combined supernatants were then centrifuged at 27,000 x g for 20 min and the resulting pellet resuspended in 5 ml TE buffer plus protease inhibitors to obtain a crude plasma membrane (P_1) fraction. Supernatants were then centrifuged at 200,000 x g for 90 min to yield 'light' membrane (V_1) and high speed supernatant (S_2) fractions. All subcellular fractions

were resuspended in 0.5 ml of KGEH buffer (139 mM potassium glutamate, 4 mM $MgCl_2$, 10 mM EGTA, and 30 mM HEPES, pH 7.4) prior to analysis.

Western Blot Analysis. Aliquots (30 µg of protein) of subcellular fractions were mixed with Laemmli SDS-sample buffer and resolved by SDS-PAGE on a 10% Tris-HCl polyacrylamide gel (Biorad, Hercules, CA). Proteins were transferred to a nitrocellulose membrane (0.45 µm Nitrobind; GE Osmonics, Minnetonka, MN) and non-specific binding sites were blocked by incubation in tris-buffered saline (pH 7.4) containing 0.1 % Tween (TBS-T) with 2.5% non-fat milk and 2.5% BSA for 1 h at 22^{o}C. Primary antibodies were diluted in blocking solution and incubated with membranes for 12 h at 4^{o}C. Excess primary antibody was removed by washing the membranes 3 x 10 min in TBS-T. The blots were then incubated in the appropriate HRP-conjugated secondary antibody (diluted as specified) in blocking solution for 1 hr at 22^{o}C and subsequently washed 3 more times for 10 min in TBS-T. Immunoreactive proteins were detected with ECL (Western Lightening; Perkin Elmer, Waltham, MA) and images were captured with a EpiChemi3 darkroom (UVP, Inc., Upland, CA).

Radioligand binding. Subcellular fractions of SH-SY5Y cells were resuspended in KGEH buffer (139 mM potassium glutamate, 4 mM $MgCl_2$, 10 mM EGTA and 30 mM HEPES, pH 7.4) and incubated in a 2 ml volume with 0.5 nM [^{3}H]QNB at 37^{o} C for 90 min. Non-specific binding was determined as that unaffected by inclusion of 12.5 µM atropine. Reactions were terminated by rapid filtration through Whatman GF / B

filters and radioactivity determined after the addition of 7.5 ml of Universol scintillation fluid. Specific [^{3}H]QNB binding was calculated as fmol bound / mg of protein.

Data Analysis. All experiments shown were performed in duplicate or triplicate and repeated at least three times. Values quoted are given as means ± S.E.M. for the number (n) of independent experiments indicated. Quantification of Western blot bands was performed using Scion Image software (Scion Corporation, Frederick, MD). A paired two-tailed Student's 't' test was used to evaluate differences between two experimental groups (level of significance, $p < 0.05$). Repeated measures analysis of variance (ANOVA) was routinely followed by a Bonferroni multiple comparisons test for statistical significance of differences between multiple groups. A Dunnett's multiple comparisons test was used when the only analysis of interest was the comparison of multiple groups to one control value. EC_{50} values were obtained using Prism 5.01 (GraphPad Software Inc., San Diego, CA). Kinetic analysis of D-aspartate uptake was fit to the sum of carrier-mediated transport and diffusional components using a non-linear least squares approximation, as published previously (Foster et al., 2009), according to the following equation (Equation 1):

$$V = \frac{V_{max} \bullet [S]}{K_m + [S]} + k_{diff} \bullet [S]$$

Kinetic values quoted are the best-fit values ± SE as derived using Prism 5.01 (Graphpad Software Inc.). In this equation, V is the observed rate of D-aspartate

uptake (nmol / mg protein / min), V_{max} is the maximum rate of uptake, K_m is the aspartate affinity binding constant (μM) for the saturable component of uptake, k_{diff} is the rate constant for the non-saturable, diffusion-mediated, influx (ml / mg protein / min), and S is the concentration of extracellular D-aspartate (μM). From this equation, the relative contributions of both the saturable, carrier-mediated, component, and the non-saturable, diffusional, component can be determined.

Results

Uptake of D-aspartate into SH-SY5Y neuroblastoma cells is facilitated by muscarinic receptor activation and attenuated by hyposmolarity. When SH-SY5Y cells were incubated at 37^{o}C in isotonic buffer (290 mOsM) that contained 10 μM D-aspartate, the uptake of D-aspartate (used as a marker for L-glutamate), proceeded linearly for at least 45 min at a rate of 12 ± 1 pmol aspartate / mg protein / min (Fig. 4.1A). D-aspartate uptake was dependent upon the presence of NaCl (>85% inhibition in the absence of added NaCl: Fig. 4.1B) and was inhibited by 97 ± 2% when incubations were conducted at 4^{o}C (n=3). Analysis of SH-SY5Y cells indicated an endogenous glutamate concentration of 100 ± 13 nmol / mg protein (n=5). Given that the intracellular water space in SH-SY5Y cells is 8.5 μl / mg protein (Foster et al., 2008), an intracellular concentration of glutamate of ~12 mM can be calculated, a value similar to that observed for whole brain (McIlwain and Bachelard, 1971). Inclusion of the muscarinic cholinergic agonist, Oxo-M (100 μM), significantly increased the rate of D-aspartate uptake to 275% of control (33 ± 1 pmol aspartate / mg protein / min) when compared to that observed under basal isotonic conditions ($p < 0.05$ by ANOVA with a post hoc Bonferroni Test: Fig. 4.1A). Inclusion of 10 μM atropine fully prevented the ability of Oxo-M to stimulate D-aspartate uptake (data not shown). Both basal- and Oxo-M- stimulated D-aspartate uptake were inhibited (~60%) by inclusion of 100 μM L-cysteine, an amino acid that is co-transported by EAATs, in particular EAAT3 (Zerangue and Kavanaugh, 1996; Aoyama et al., 2006). In contrast, inclusion of either L-alanine or

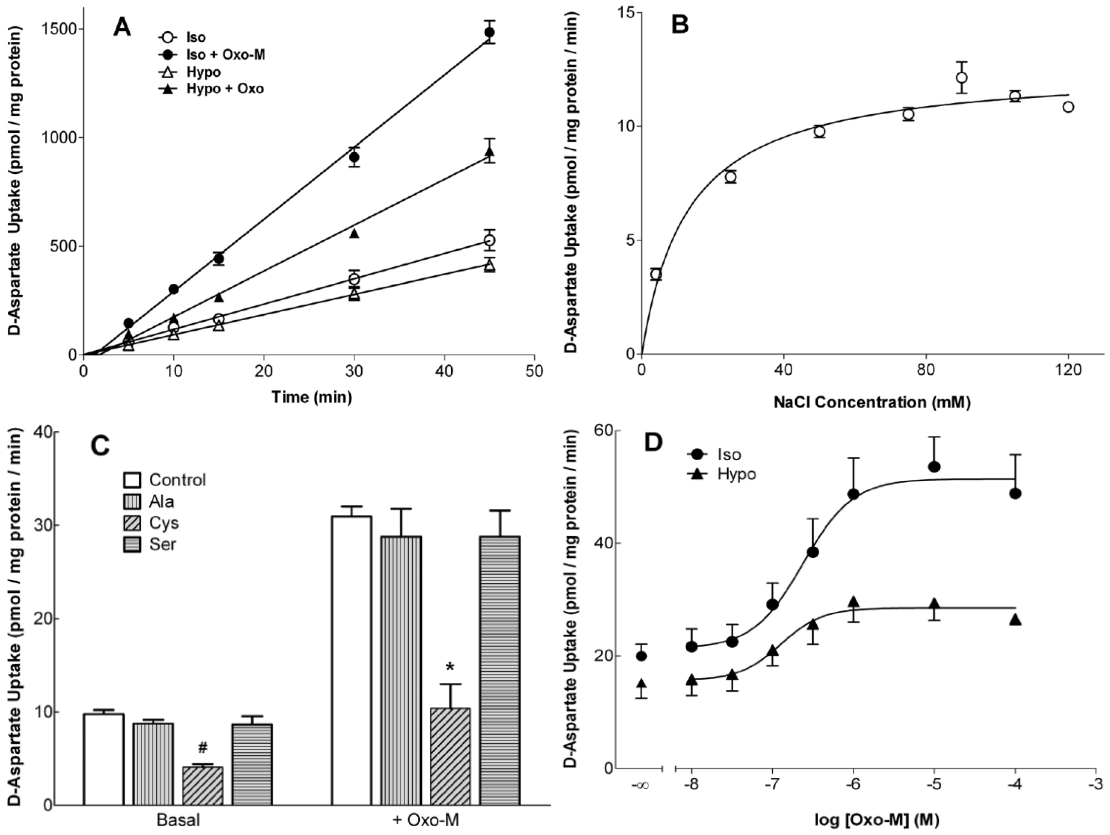

Figure 4.1 Characteristics of basal- and Oxo-M-stimulated D-aspartate uptake in SH-SY5Y cells. (A) Cells were incubated in isotonic buffer (Iso; 290 mOsM) in the absence (○) or presence (●) of 100 μM Oxo-M, or hypotonic buffer (Hypo; 230 mOsM) with (▲) or without (Δ) addition of Oxo-M. All incubations contained 10 μM D-aspartate; reactions were terminated at the times indicated and ^{3}H-D-aspartate uptake was monitored. Results are the means ± SEM of 3 independent experiments each performed in duplicate. Where error bars are absent, the SEM fell within the symbol. Rates of D-aspartate influx were calculated from linear regression analyses of the data. (B) Cells were incubated for 45 min, in the presence of NaCl at the concentrations indicated, and basal uptake of D-aspartate monitored. Where NaCl is reduced, sucrose was substituted in order to maintain isotonicity. Results are the means ± SEM for three independent experiments. (C) Cells were incubated under isotonic conditions for 45 min in the absence or presence of Oxo-M (100μM) with 100μM concentrations of L-cysteine (L-Cys), L-serine (L-Ser) or L-alanine (L-Ala). Results are the means ± SEM for three independent experiments. $p<0.05$ different from D-aspartate uptake measured under control conditions in the absence (#) or presence (*) of Oxo-M (by repeated measures ANOVA with a post-hoc Bonferroni multiple comparisons test). (D) Dose-response relationship for Oxo-M-mediated stimulation of D-aspartate uptake under isotonic (●) or hypotonic (▲) conditions (290 and 230 mOsM, respectively). Reactions were terminated after 10 min and D-aspartate uptake monitored. Results are the means ± SEM for three independent experiments.

L-serine, two neutral amino acids, was without effect (Fig. 4.1C). The rates of both basal- and Oxo-M-stimulated D-aspartate uptake were attenuated by a modest (21%) reduction in osmolarity of the incubation medium (Fig. 4.1A). Hypotonicity reduced the rate of Oxo-M-stimulated D-aspartate uptake by ~40-50% but had no effect on the agonist EC_{50} values (0.24 and 0.13 μM under isotonic and hypotonic conditions, respectively; Fig. 4.1D). Hypotonicity consistently resulted in ~20% reduction in the basal rate of D-aspartate uptake during the course of this study (14 ± 1 vs. 11 ±1 pmol / mg protein / min under isotonic and hypotonic conditions, respectively, n= 36, $p<0.001$). The corresponding rates for D-aspartate uptake in the presence of Oxo-M under isotonic and hypotonic conditions were 40 ± 2 and 24 ± 1 pmol / mg protein, (n=36, $p<0.001$). The possibility that reversal of EAAT contributes to the inhibition of D-aspartate uptake observed under hypoosmotic conditions can be excluded since inclusion of 200 μM TBOA, a non-transportable inhibitor of EAATs, had no effect on either the basal- or Oxo-M-stimulated D-aspartate efflux from SH-SY5Y cells (data not shown).

The sensitivity of D-aspartate uptake to changes in osmolarity (340-200 mOsM), was monitored under conditions of a constant NaCl concentration (Fig. 4.2A). Both basal- and Oxo-M-stimulated uptake were attenuated as osmolarity was reduced. Although the basal uptake was not significantly reduced from the value obtained under isotonic conditions in this limited series of experiments, a statistically significant attenuation (~40%) of Oxo-M-stimulated uptake was observed following a reduction in osmolarity of 16%, whereas a 74% loss of D-aspartate uptake was observed when the osmolarity was reduced by 31%. In all subsequent experiments,

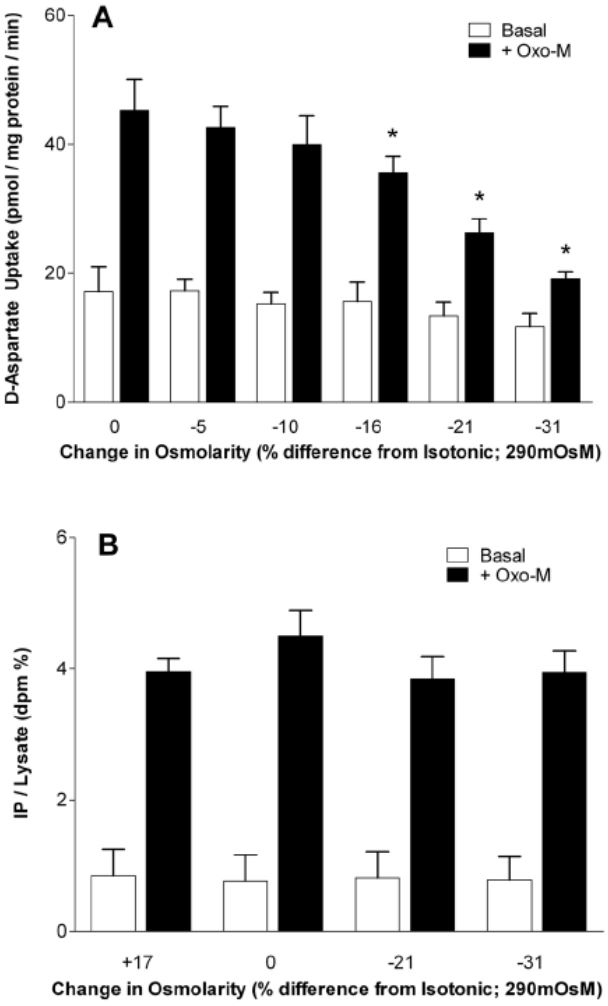

Figure 4.2 Effect of osmolarity on D-aspartate uptake and mAChR signaling. (A) Cells were incubated in buffers of the osmolarity indicated (isotonic buffer = 290 mOsM) in either the absence (open bars) or presence (closed bars) of 100 μM Oxo-M. The osmolarity of the buffers was adjusted under conditions of a fixed NaCl concentration (90 mM), by the addition of sucrose. Reactions were terminated after 10 min and D-aspartate uptake monitored. Results are the means ± SEM of 4 independent experiments each performed in duplicate. *, $p<0.05$ different from D-aspartate uptake measured under isotonic conditions (290 mOsm) in the presence of Oxo-M (by repeated measures ANOVA followed by a Bonferroni multiple comparisons test). (B) Cells that had been prelabeled for 48 hours with ^{3}H-inositol were treated for 10 min in buffers of the osmolarity indicated in either the absence (open bars) or presence (closed bars) of 100 μM Oxo-M. Reactions were terminated by the addition of trichloroacetic acid and the accumulation of radiolabeled inositol phosphates (IP) was monitored as an index of stimulated phosphoinositide turnover. Results are expressed as inositol phosphate (IP) released / total soluble radioactivity in cell lysates (IP / lysate; dpm %) and are the means ± S.E.M. for 3 independent experiments, each performed in triplicate.

an osmolarity of 230 mOsM (a 21% reduction from isotonic) was routinely employed. To examine the possibility that these reductions in mAChR-mediated D-aspartate uptake observed under hypoosmotic conditions were secondary to an attenuation of cell signaling events, both basal- and mAChR-stimulated phosphoinositide turnover were monitored over a wide range of osmolarities. Neither hyper- nor hypotonic conditions influenced the ability of Oxo-M to enhance phosphoinositide turnover, a result that indicates that mAChR-mediated signaling events are not directly regulated by osmolarity (Fig. 4.2B).

Osmosensitive reductions in Oxo-M-stimulated D-aspartate uptake are readily reversible. To address the possibility that exposure of the cells to hypotonic media resulted in deleterious effects, we determined whether the osmolarity-dependent reductions in agonist-stimulated D-aspartate uptake could be reversed. Cells were first preincubated for 10 min in either isotonic or hypotonic buffer in the absence or presence of 100 μM Oxo-M. Cells were then rapidly washed with isotonic buffer and D-aspartate uptake monitored for 20 min under either isotonic or hypotonic conditions, in the presence or absence of the agonist (Fig. 4.3). Cells that had been preincubated in hypotonic buffer and then subsequently exposed to isotonic buffer (condition B), exhibited rates of D-aspartate uptake that were comparable to cells that had been exposed to isotonic conditions during both the preincubation and incubation phases (condition C). In contrast, cells that were maintained under hypotonic conditions during both preincubation and incubation phases (condition A) exhibited rates of D-aspartate uptake that were 40% lower than cells incubated

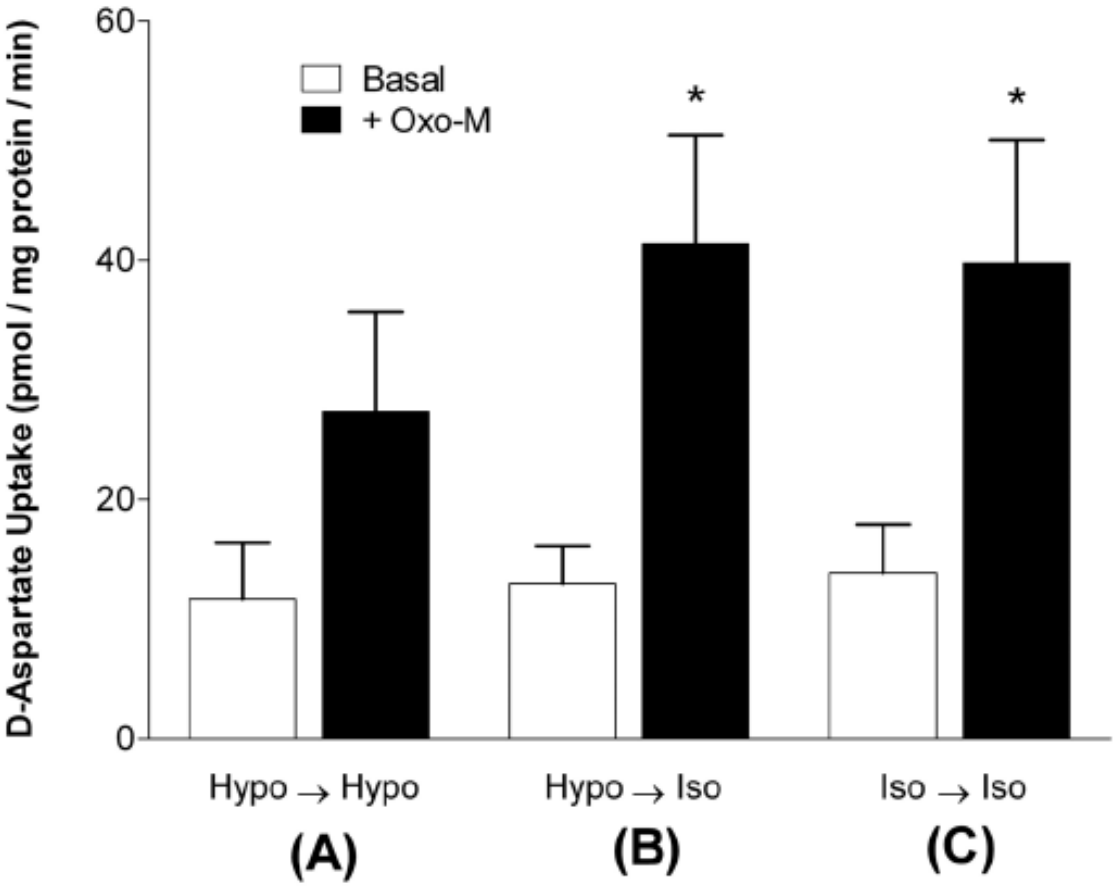

Figure 4.3 Reversibility of osmolarity-mediated inhibition of D-aspartate uptake. Cells were first preincubated for 10 min in either hypotonic buffer (Hypo; 230 mOsM, conditions A and B) or isotonic buffer (Iso; 290 mOsM, condition C) in the presence or absence of 100 μM Oxo-M. Cells were then washed twice with 2 ml of buffer (osmolarity of wash buffer matched to that of the pretreatment buffer) and incubated with 10 μM ^{3}H-D-aspartate for an additional 20 min in 5 ml of either hypotonic (condition A) or isotonic buffers (conditions B and C) in the presence (closed bars) or absence (open bars) of Oxo-M (100 μM). Results are expressed as D-aspartate uptake (pmol / mg protein / min) and are the means ± SEM of 4 independent experiments, each performed in triplicate. *, $p<0.05$ different from D-aspartate uptake measured under condition A in the presence of Oxo-M (by repeated measures ANOVA followed by a Bonferroni multiple comparisons test).

under conditions (B) or (C). These results suggest that tonicity-induced changes in D-aspartate uptake are readily reversible.

Substrate concentration-dependence of D-aspartate uptake. To determine whether changes in D-aspartate uptake observed under isotonic or hypotonic conditions reflected changes in the V_{max} and / or K_m values for D-aspartate uptake, the rate of D-aspartate uptake was monitored in the presence of increasing substrate concentrations. Rectangular hyperbolic dose-response plots were obtained for D-aspartate uptake under both isotonic and hypotonic conditions (Fig. 4.4). The diffusional (non-saturable) component of D-aspartate influx was negligible under isotonic or hypotonic conditions in either the absence or presence of Oxo-M (< 3.5% of total). The K_m values for D-aspartate uptake (2.4 - 3.2 μM) were similar under isotonic and hypotonic conditions in either the presence or absence of Oxo-M. However, under both isotonic and hypotonic conditions, the inclusion of Oxo-M significantly increased the V_{max} values for D-aspartate uptake. Thus under isotonic conditions, the V_{max} increased from 21 ± 1 to 63 ± 5 pmol / mg protein / min, whereas the comparable values obtained under hypotonic conditions were 15 ± 2 and 41 ± 5 pmol / mg protein / min ($p < 0.05$ by ANOVA). These results suggest that the increase in D-aspartate uptake observed in the presence of Oxo-M and the reduction in uptake mediated by hyposmolarity occur primarily via changes in the V_{max} for uptake.

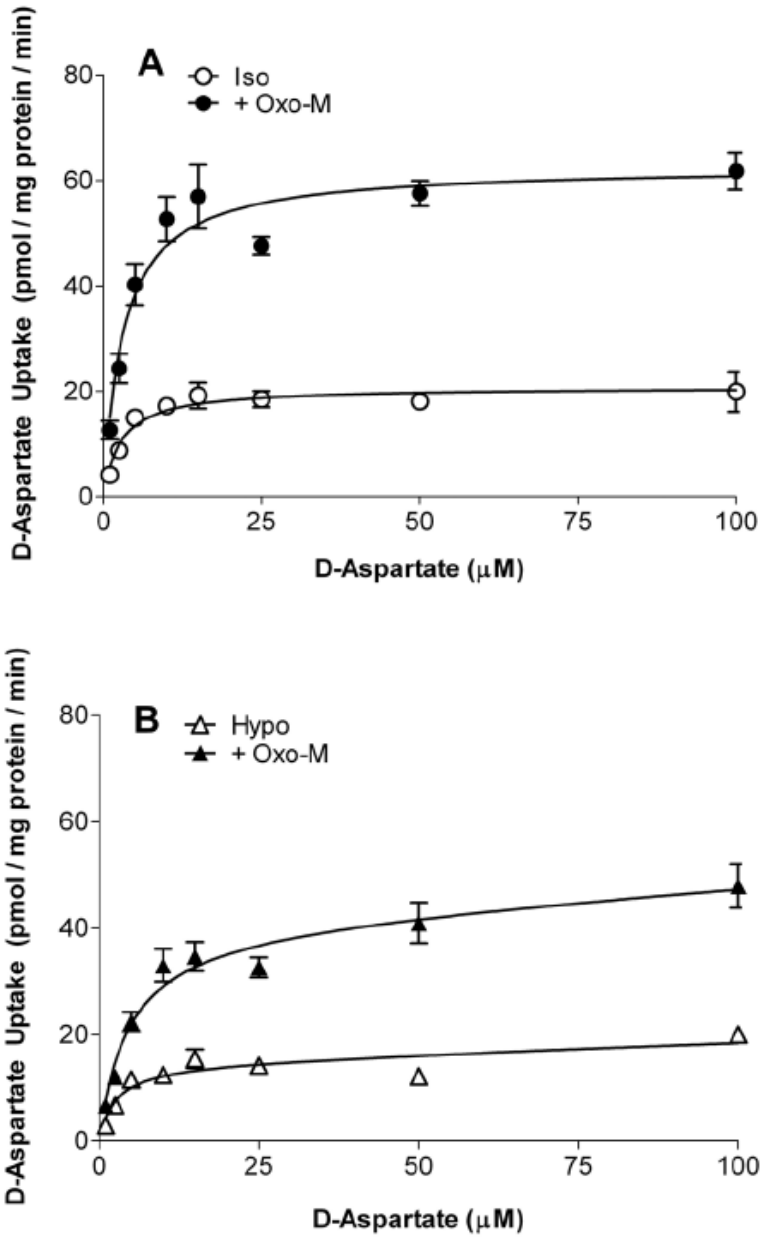

Figure 4.4 Substrate concentration-dependence of D-aspartate uptake. SH-SY5Y cells were incubated in either (A) isotonic (Iso; 290 mOsM) or (B) hypotonic (Hypo; 230 mOsM) buffer in the absence (open symbols) or presence (closed symbols) of 100 μM Oxo-M at the D-aspartate concentrations indicated. Reactions were terminated after 10 min and D-aspartate uptake was determined. Results are the means ± SEM of four independent experiments each performed in duplicate. Where error bars are absent, the SEM fell within the symbol. Kinetic parameters were obtained from equation 1, as described in the Materials and Methods.

Agonist-mediated increases in D-aspartate uptake are receptor-specific in SH-SY5Y cells. In addition to the mAChR, several GPCRs including the protease-activated receptor (PAR), sphingosine-1-phosphate (S1P) and lysophosphatidic (LPA) receptors have previously been demonstrated to regulate both the uptake and efflux of organic and inorganic osmolytes in SH-SY5Y cells (Foster et al., 2008; Foster et al., 2009). To determine whether these receptors also facilitate D-aspartate uptake, SH-SY5Y cells were exposed to isotonic or hypotonic buffer in the absence or presence of 100 μM Oxo-M, 1.25 nM thrombin (a PAR agonist), 5 μM S1P or 10 μM LPA (Fig. 4.5). The addition of thrombin or S1P had little or no effect under either isotonic or hypotonic conditions, whereas inclusion of LPA resulted in a small, but statistically significant, increase in D-aspartate uptake under isotonic, but not hypotonic, conditions (Fig. 4.5, $n = 4$, $p < 0.05$ by repeated measures ANOVA). In a separate series of experiments, the addition of 1 nM endothelin under isotonic conditions resulted in a statistically significant increase in the uptake of D-aspartate that was 36 ± 3 % of that observed in the presence of Oxo-M ($n=4$, $p<0.05$).

Pharmacological characterization of basal- and Oxo-M-stimulated D-aspartate uptake. Three distinct subtypes of excitatory amino acid transporters (EAAT1-3) have been reported to be present in SH-SY5Y cells (Sala et al., 2005). To examine the subtype(s) of transporter responsible for D-aspartate uptake under basal- or agonist-stimulated conditions, the following pharmacological inhibitors were utilized: TBOA, a non-transportable inhibitor of EAAT1-5 (200 μM), L3HA, a transportable inhibitor of EAAT1-4 (200 μM), DHK, a non-transportable inhibitor of EAAT2

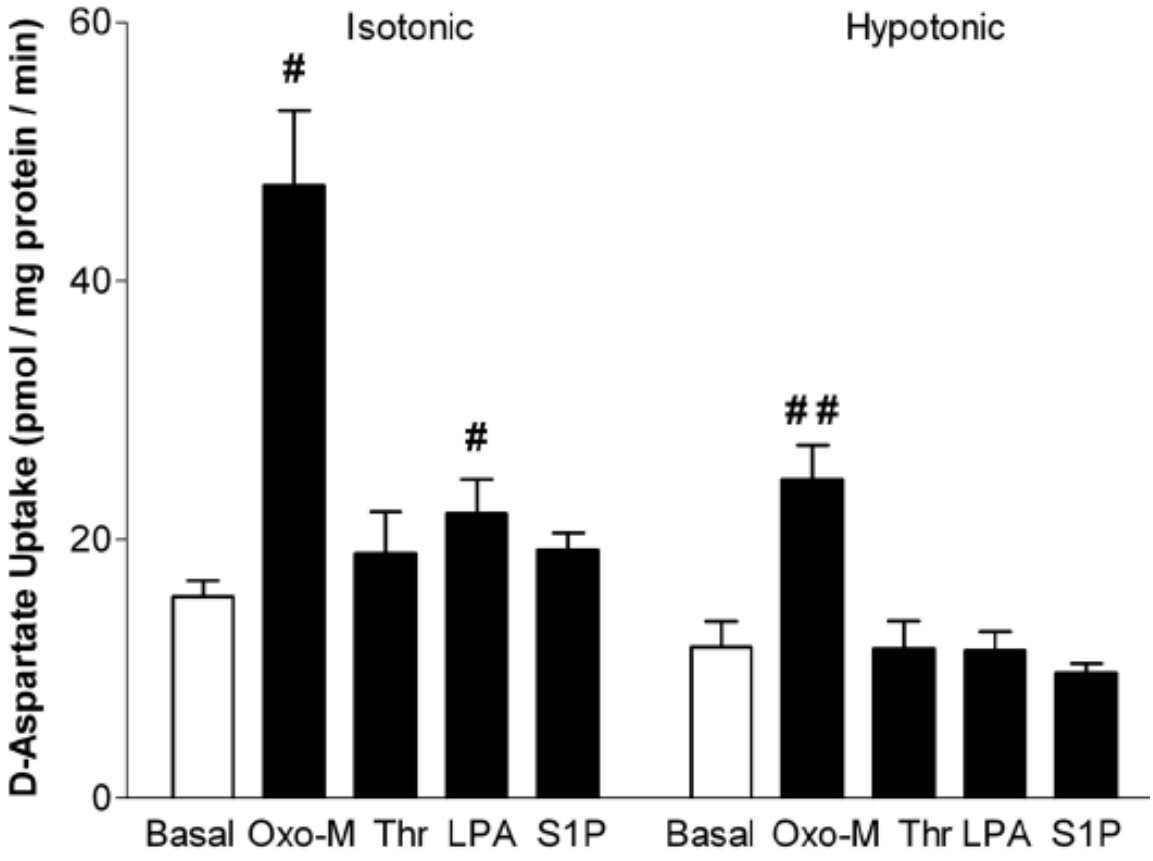

Figure 4.5 Agonist-mediated increases in D-aspartate uptake are receptor-specific. Cells were incubated under either isotonic (290 mOsM) or hypotonic (230 mOsM) conditions in absence or presence of 100 μM Oxo-M, 1.25 nM thrombin (Thr), 5 μM sphingosine-1-phosphate (S1P) or 10 μM lysophosphatidic acid (LPA). Reactions were terminated after 10 min and D-aspartate uptake was monitored. Results are the means ± SEM of 4 independent experiments each performed in duplicate. $p<0.05$ different from D-aspartate uptake observed under isotonic (#) or hypotonic (##) basal conditions (by repeated measures ANOVA followed by a Bonferroni multiple comparisons test).

(200 μM) or LβBA, a non-transportable inhibitor of EAAT3 (and to a lesser extent, EAAT1;0.3-100 μM). Addition of DHK had little effect on either basal (Fig. 4.6A) or Oxo-M-stimulated (Fig. 4.6B) D-aspartate uptake indicating that EAAT2 is unlikely to play a significant role in either of these processes. Inclusion of either TBOA or L3HA essentially completely abolished Oxo-M-stimulated D-aspartate uptake (~95%) and severely attenuated basal uptake (~75%) indicating that the uptake is mediated via EAAT1 and / or EAAT3. To potentially differentiate between the involvement of EAAT1 and EAAT3, a dose-inhibition curve was constructed for the EAAT3-selective inhibitor, LβBA. This agent exhibits a 6-fold selectivity for EAAT3 over EAAT1 (Ki values of 2 and 12 μM, respectively, Esslinger et al., 2005). D-aspartate uptake under both basal- and Oxo-M-stimulated conditions was potently inhibited by LβBA (IC_{50} values= 8.6 and 2.6 μM, respectively) with corresponding K_i values calculated from the Cheng-Prusoff equation (Cheng and Prusoff, 1973) of 1.8 μM and 0.6 μM. Taken collectively, these results suggest that EAAT3 plays a major role in the basal- and Oxo-M-stimulated uptake of D-aspartate into SH-SY5Y cells.

Endothelin-1-mediated stimulation of D-aspartate uptake in C6 glioma cells is also osmosensitive. Previous reports have demonstrated that activation of endothelin-1 receptors in C6 glioma cells increases high affinity D-aspartate uptake, a process mediated by the EAAT3 transporter (Najimi et al., 2005). To determine whether D-aspartate uptake in C6 glioma cells is also sensitive to changes in osmolarity, uptake of D-aspartate was measured under either isotonic or hypotonic conditions, in the presence or absence of 1 nM endothelin. The rate of D-aspartate

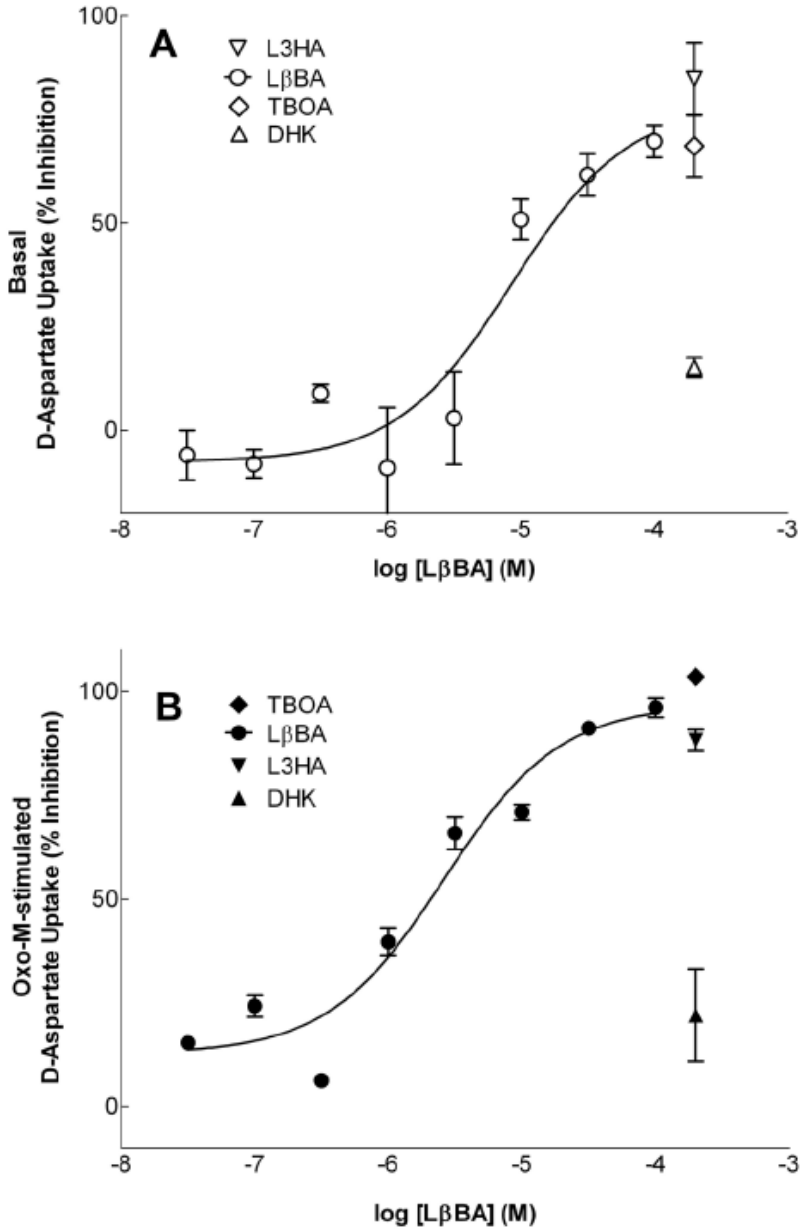

Figure 4.6 Effect of EAAT inhibitors on basal- and Oxo-M-stimulated D-aspartate uptake. Cells were incubated in isotonic buffer (290 mOsM) in the absence (A) or presence (B) of 100 µM Oxo-M with or without the EAAT inhibitors: DL-threo-β-benzyloxyaspartic acid (TBOA), L-β-threo-benzyl-aspartate (LβBA), L-(-)-*threo*-3-hydroxyaspartic acid (L3HA), or dihydrokainic acid (DHK), at the concentrations indicated. Reactions were terminated after 10 min and results are presented as (A) percentage inhibition of basal D-aspartate uptake or (B) percentage inhibition of Oxo-M-stimulated D-aspartate uptake and are the means ± SEM of 3-6 independent experiments each performed in duplicate. LβBA inhibited basal D-aspartate uptake with an IC_{50} of 8.6 μM and attenuated Oxo-M-stimulated D-aspartate uptake with an IC_{50} of 2.6 μM.

uptake under basal isotonic conditions (298 ± 7 pmol / mg protein / min, n=3) was ~20 fold greater than that observed in SH-SY5Y cells. Addition of ET-1 under isotonic conditions caused a significant (65%) stimulation of D-aspartate uptake (492 ± 33 pmol / mg protein / min; n=3, $p<0.05$ by repeated measures ANOVA), as previously reported. Under hypotonic conditions a small yet significant decrease in basal uptake was observed (~14% reduction compared to basal isotonic conditions). Although the addition of ET-1 under hypotonic conditions increased D-aspartate uptake, the degree of this stimulation was significantly inhibited (~45%) when compared to ET-1-mediated uptake under isotonic conditons (n=3, $p<0.05$ by repeated measures ANOVA).

Oxo-M-mediated stimulation of D-aspartate uptake in SH-SY5Y cells occurs via PKC- and Ca^{2+}-dependent mechanisms. Agonist occupancy of mAChRs in SH-SY5Y cells is known to lead to the activation of PKC and mobilization of intracellular Ca^{2+} (Cioffi and Fisher, 1990). To evaluate the role for PKC and Ca^{2+} in Oxo-M-stimulated D-aspartate uptake, SH-SY5Y cells were incubated under isotonic or hypotonic conditions in the absence or presence of 100 μM Oxo-M or optimal concentrations of either PMA (100 nM) or ionomycin (1 μM). Addition of PMA significantly increased D-aspartate uptake under both isotonic and hypotonic conditions ($p < 0.05$ different from control basal by repeated measures ANOVA). Addition of ionomycin also resulted in increased D-aspartate uptake under isotonic conditions, but not under hypoosmotic conditions (Fig. 4.7B). In the presence of both PMA and ionomycin, the rate of D-aspartate uptake under isotonic or hypotonic

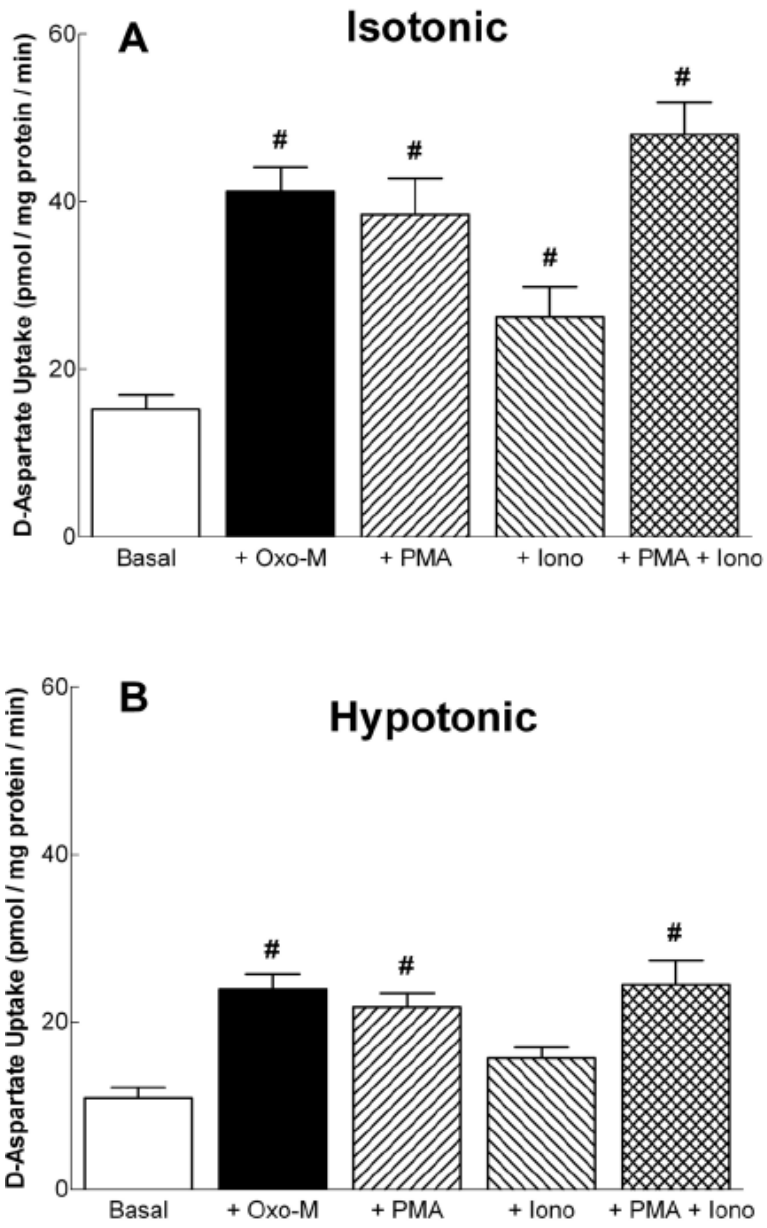

Figure 4.7 Agents that either activate PKC or increase intracellular Ca^{2+} concentrations mimic the ability of Oxo-M to induce D-aspartate uptake. D-aspartate uptake was monitored in SH-SY5Y cells after 10 min of incubation under (A) isotonic (290 mOsM) or (B) hypotonic (230 mOsM) conditions in the absence or presence of 100 µM Oxo-M, 100nM phorbol 12-myristate 13-acetate (PMA), or 1 µM ionomycin (Iono). Results shown are the means ± SEM of 3-4 independent experiments each performed in duplicate. #, $p<0.05$ different from D-aspartate uptake observed under basal conditions (by repeated measures ANOVA followed by Dunnett's multiple comparisons test).

conditions was comparable to that observed in the presence of Oxo-M (Fig. 4.7A,B). To further examine a role for PKC in the mediation of Oxo-M-stimulated D-aspartate uptake, cells were preincubated for 10 min with either vehicle (DMSO) or 2.5 μM of BIM (a broad spectrum PKC inhibitor) prior to the addition of either Oxo-M or PMA. Although BIM had no effect on basal D-aspartate uptake, it fully blocked the ability of PMA to stimulate uptake but only partially attenuated (30-45%) the ability of Oxo-M to enhance D-aspartate uptake, under either isotonic or hypotonic conditions (Fig. 4.8A,B: $p < 0.05$ different from control + Oxo-M by repeated measures ANOVA). In contrast, the Ca^{2+} dependence of mAChR-stimulated D-aspartate uptake differed under isotonic and hypotonic conditions. Thus under isotonic conditions, removal of extracellular Ca^{2+} resulted in a 60% inhibition of mAChR-mediated D-aspartate uptake (Fig. 4.9A) whereas little or no effect was observed under hypotonic conditions (Fig. 4.9B). Depletion of intracellular stores of Ca^{2+} with thapsigargin resulted in a small inhibitory effect on mAChR-mediated D-aspartate uptake under both isotonic and hypoosmotic conditions (Fig. 4.9A,B). The combination of inhibition of PKC with BIM, along with depletion of intra-and extracellular Ca^{2+}, essentially abolished the ability of mAChR activation to stimulate D-aspartate uptake under both isotonic and hypoosmotic conditions. (Fig. 4.9C,D).

mAChR-stimulated D-aspartate uptake in SH-SY5Y cells is dependent upon PI 3-kinase activity and an intact cytoskeleton. There have been conflicting reports of the involvement of PI3K and cytoskeleton in the regulation of D-aspartate uptake (Najimi et al., 2002; Bianchi et al., 2006). Thus, the role, if any, played by these

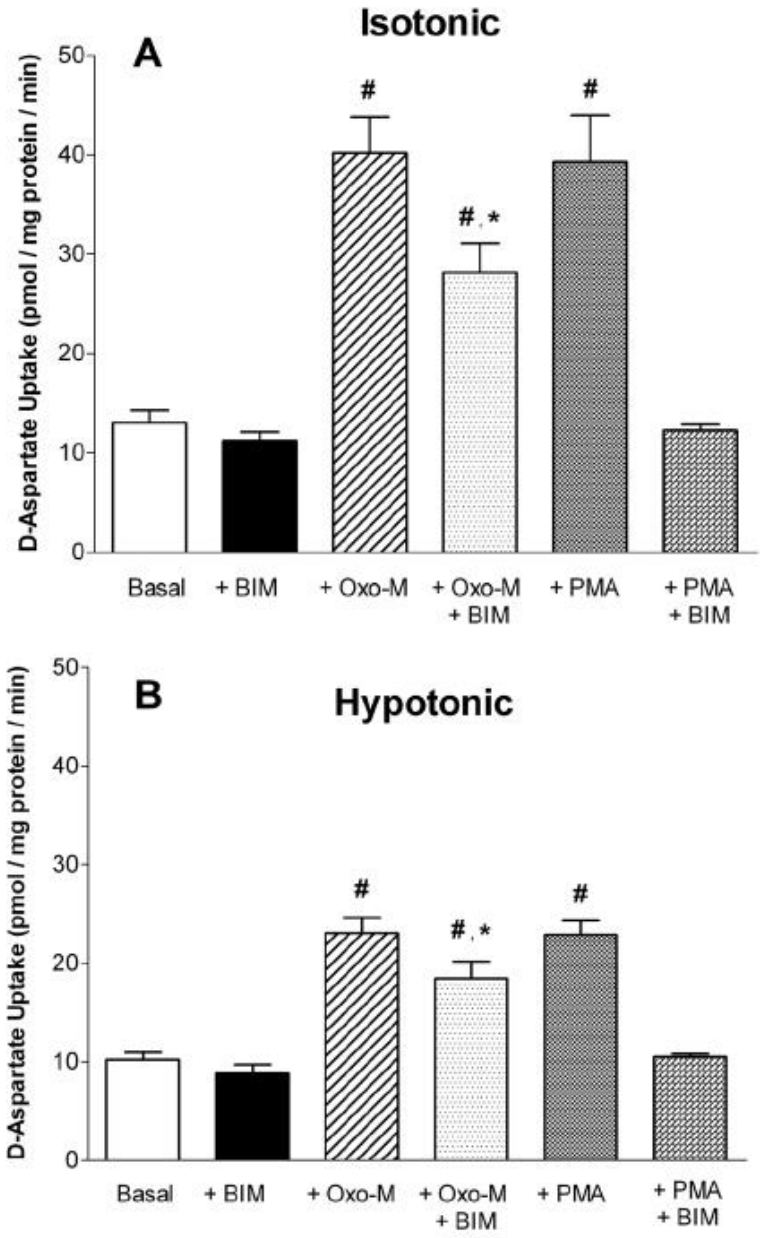

Figure 4.8 Inhibition of PKC attenuates both Oxo-M- and PMA-stimulated D-aspartate uptake. Cells were incubated under either (A) isotonic (290 mOsM) or (B) hypotonic (230 mOsM) conditions in the absence or presence of 100 µM Oxo-M, 100 nM phorbol 12-myristate 13-acetate (PMA) or 2.5 µM bisindolylmaleimide (BIM), as indicated. All cells treated with BIM received a 10 min pretreatment in isotonic buffer containing 2.5 µM BIM. Reactions were terminated after 10 min and results are expressed as D-aspartate uptake (pmol / min / mg protein) and are the means ± SEM of 3 independent experiments each performed in duplicate. $p<0.05$ different from D-aspartate uptake observed under basal (#) or Oxo-M-treated (*) conditions (by repeated measures ANOVA followed by a Bonferroni multiple comparisons test).

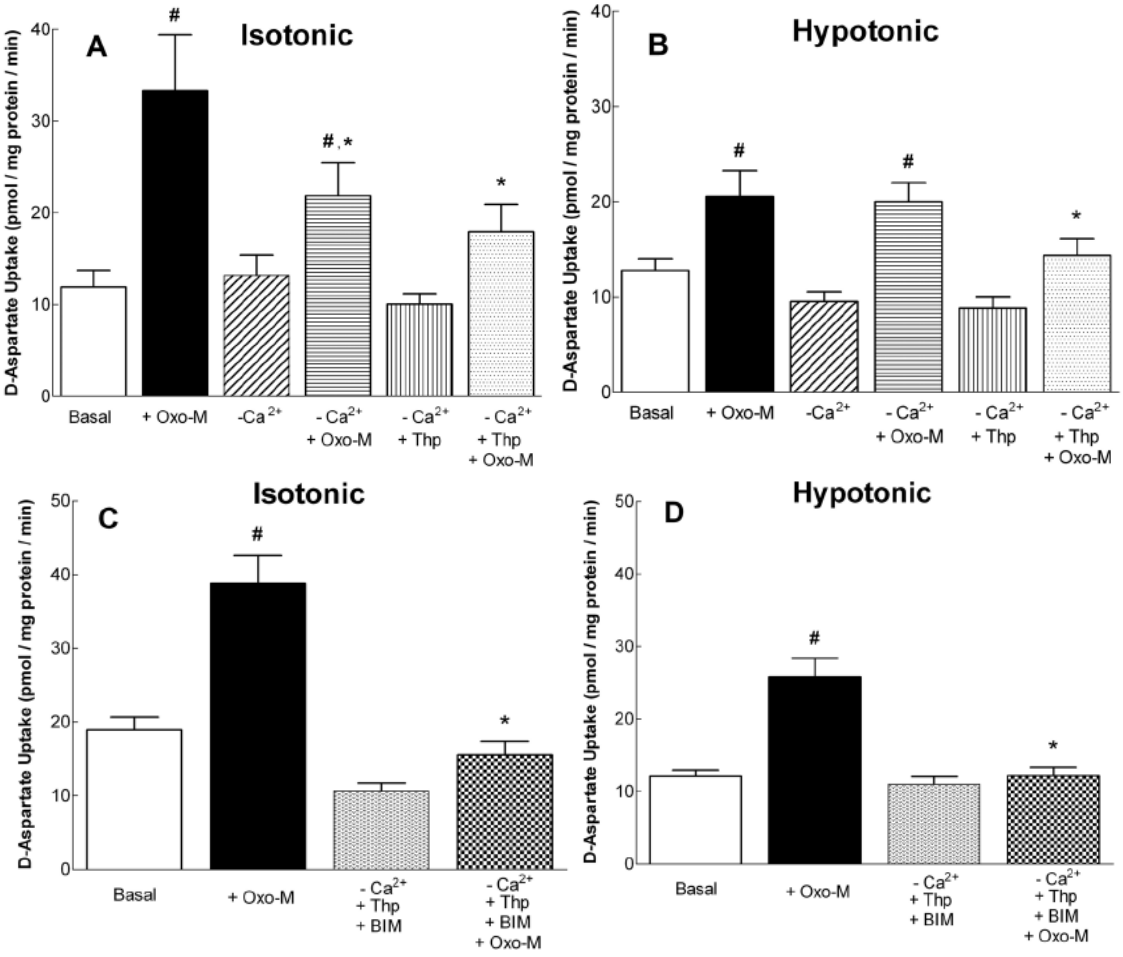

Figure 4.9 The role of extra- and intracellular Ca^{2+} in Oxo-M-stimulated D-aspartate uptake. Cells were incubated under either (A) isotonic (290 mOsM), or (B) hypotonic (230 mOsM) conditions in the absence (-Ca^{2+}; Ca^{2+} was omitted from buffer and 50 μM EGTA was added) or presence of extracellular Ca^{2+} with or without the addition of 100 μM Oxo-M. Some cells were pre-incubated for 15 min in isotonic buffer containing 1 μM thapsigargin (Thp) to deplete intracellular pools of Ca^{2+} prior to measurement of D-aspartate uptake in either the absence or presence of Oxo-M. Reactions were terminated after 10 min and D-aspartate uptake was determined. In (C) and (D), cells were preincubated for 15 min with 2.5 μM BIM and 1 μM Thp (-Ca^{2+}) and then incubated under isotonic or hypotonic conditions in the absence or presence of Oxo-M. Results shown are the means ± SEM of 4 independent experiments, each performed in duplicate. $p<0.05$ different from D-aspartate uptake observed either under basal conditions (#) or in the presence of Oxo-M alone (*) (by repeated measures ANOVA followed by a Bonferroni multiple comparisons test).

components in mAChR-mediated D-aspartate was examined. Inclusion of 100 nM wortmannin reduced the basal uptake of D-aspartate under both isotonic and hypotonic conditions and significantly attenuated (35-40%) the ability of Oxo-M to facilitate uptake ($p < 0.05$ by paired student's t test: Fig. 4.10). Disruption of the actin cytoskeleton by pretreatment of the cells with 1 μM cytochalasin D (Cyto D), or alternatively, disruption of microtubule polymerization with 10 μM colchicine, had no effect on basal D-aspartate uptake but significantly attenuated Oxo-M-stimulated D-aspartate uptake (~35-55%) under isotonic conditions when added individually or in combination (Fig. 4.11A; $n = 4$, $p < 0.05$ different vs. control + Oxo-M by repeated measures ANOVA). Similar results were obtained under hypotonic conditions with the exception that basal D-aspartate uptake was attenuated in the combined presence of cytochalasin and colchicine. A combination of hypotonicity and disruption of the actin cytoskeleton and microtubules led to a >75% inhibition of Oxo-M-stimulated D-aspartate uptake in comparison to that observed under isotonic conditions alone.

Oxo-M-mediated subcellular redistribution of EAAT3. In quiescent cells, EAAT3 is predominantly localized to an intracellular location (Beart and O'Shea, 2007). Using a cell-surface biotinylation approach, activation of neurotensin, platelet derived growth factor and endothelin receptors has previously been demonstrated to result in the appearance of EAAT3 at the plasma membrane (Najimi et al., 2002; Fournier et al., 2004; Najimi et al., 2005). Given these previous findings and the pharmacological data that indicated a primary role for EAAT3 in Oxo-M-stimulated

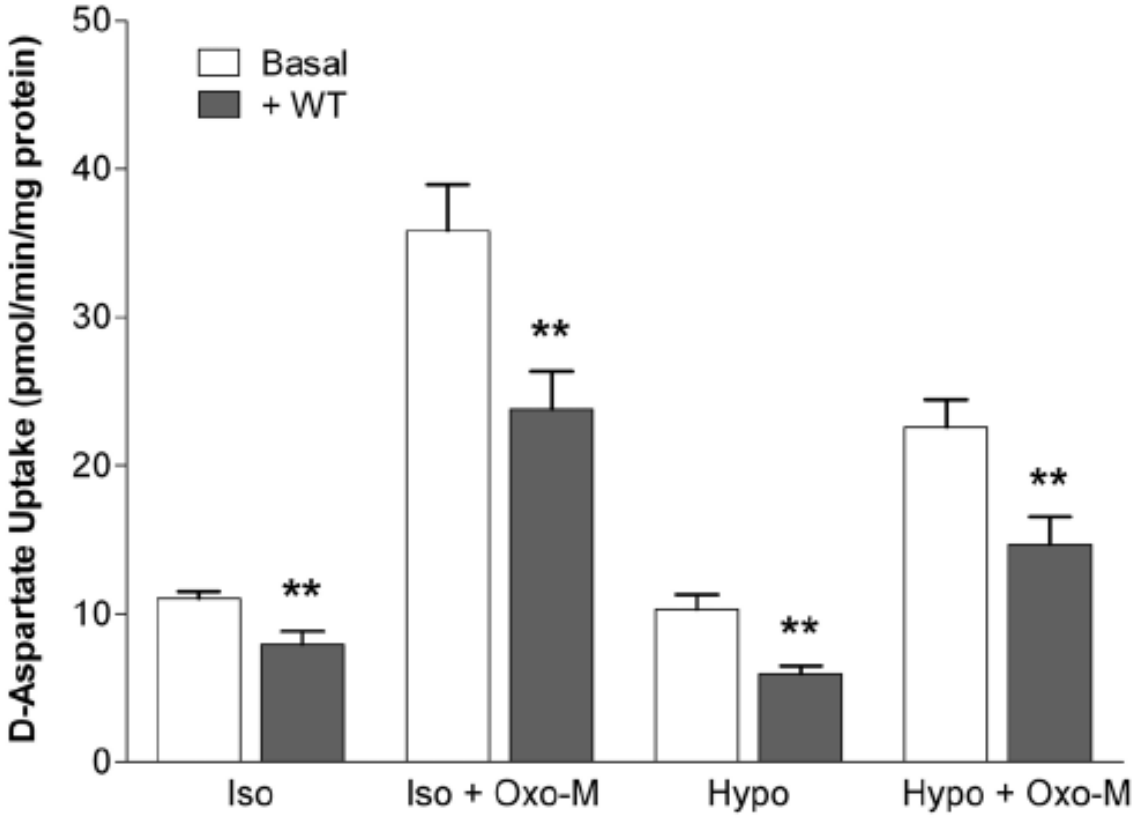

Figure 4.10 Inhibition of PI-3 kinase with wortmannin inhibits both basal- and Oxo-M- stimulated D-aspartate uptake. Cells were incubated in either isotonic (Iso; 290 mOsM) or hypotonic (Hypo; 230 mOsM) buffer in the absence or presence of 100 μM Oxo-M and 100 nM wortmannin (WT). D-aspartate uptake was monitored after 10 min. Results shown are the means ± SEM of 4 independent experiments each performed in duplicate. **, $p<0.05$ different from D-aspartate uptake observed in the absence of WT (by paired student's t test).

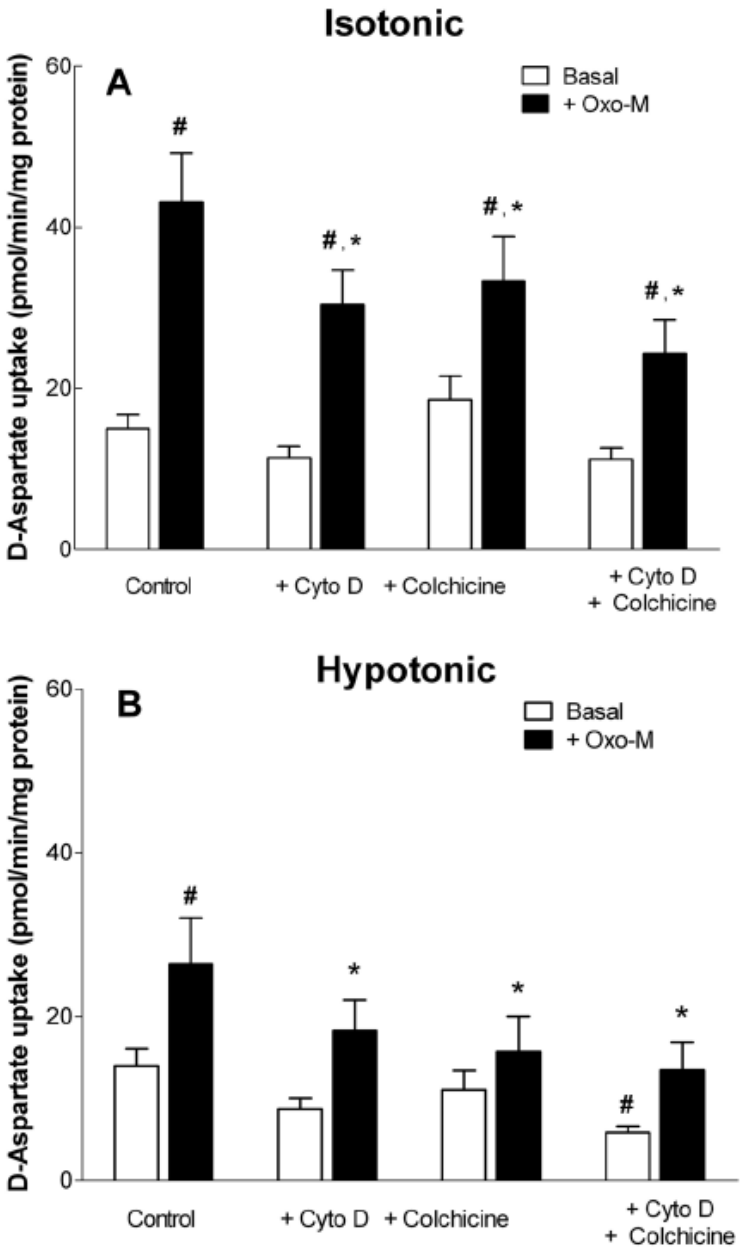

Figure 4.11 Disruption of the cytoskeleton attenuates Oxo-M-stimulated D-aspartate uptake. Cells were incubated under either (A) isotonic (290 mOsM) or (B) hypotonic (230 mOsM) conditions in the presence or absence of 100 µM Oxo-M, 1 µM cytochalasin D (Cyto D), or 10 µM colchicine for 10 min and D-aspartate uptake determined. All cells treated with Cyto D and / or colchicine were exposed to the drug(s) for 30 min in isotonic buffer prior to measurement of D-aspartate uptake. Results shown are the means ± SEM of 3 independent experiments, each performed in duplicate. $p<0.05$ different from D-aspartate uptake observed under control conditions in the absence (#) or presence (*) of Oxo-M (by repeated measures ANOVA followed by a Bonferroni multiple comparisons test).

D-aspartate uptake (Fig. 4.6), the effect of Oxo-M and hypotonicity on the subcellular localization of EAAT3 was examined using a previously characterized fractionation protocol for this cell line (Sorensen et al., 1997). (Efforts to use a biotinylation technique were unsuccessful due to the relatively low expression of EAAT3 in the SH-SY5Y cell line). Cells were treated under isotonic or hypotonic conditions ± Oxo-M and then subjected to subcellular fractionation to obtain a crude plasma membrane fraction (P_1: that is enriched in ^{3}H-ouabain binding, a marker for $Na^+ + K^+$-ATPase activity) and an endosomal fraction (V_1: that is enriched in NADPH cytochrome c reductase activity and endocytosed mAChRs). There were no significant differences in the immunoreactivities of actin or EEA1 recovered in the P_1 and V_1 fractions, respectively, across the experimental conditions examined and thus these values were routinely used as loading controls. Under basal isotonic conditions, EAAT3 immunoreactivity (detected as a major band at 66 KDa on Western blots) was ~ 4x greater in the V_1 fraction than in the P_1 fraction. However, the addition of Oxo-M increased the amount of EAAT3 immunoreactivity associated with the P_1 fraction by ~ 2 fold (different from control cells treated, $p < 0.05$ by repeated measures ANOVA). A concurrent reduction in the amount of EAAT3 detected in the V_1 fraction (~ 25%) was observed upon addition of Oxo-M under isotonic conditions, although this value did not reach statistical significance. Unexpectedly, a similar enrichment of EAAT3 immunoreactivity in the P_1 fraction (relative to basal isotonic conditions) occurred under basal hypotonic conditions and no further increase was observed in the presence of Oxo-M (Fig. 4.12B). No immunoreactivity associated with either EAAT1 or EAAT2 could be reliably detected

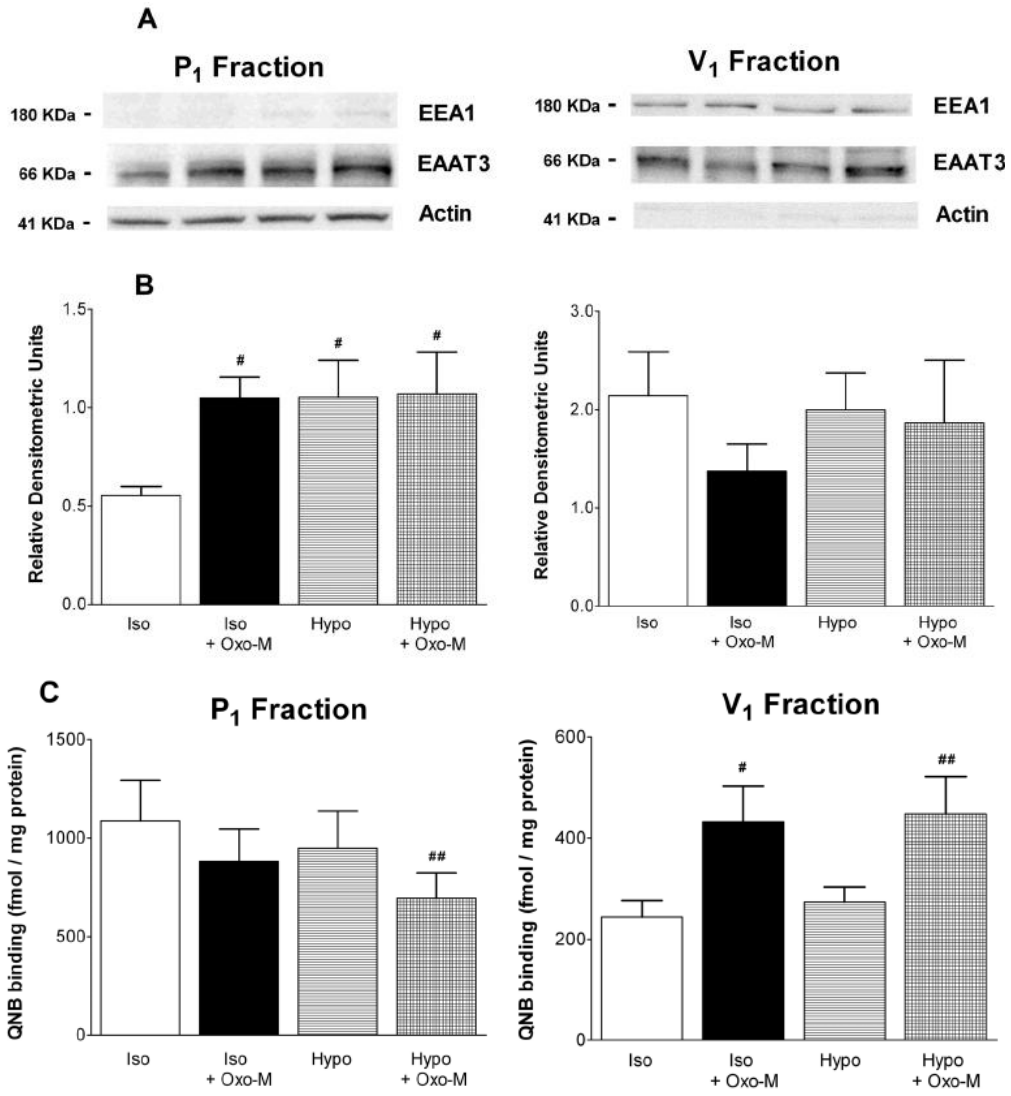

Figure 4.12 Western blot analysis of EAAT3 in subcellular fractions derived from SH-SY5Y cells incubated in the absence or presence of Oxo-M. Cells were treated for 10 min in either isotonic (Iso; 290 mOsM) or hypotonic (Hypo; 230 mOsM) buffer in the absence or presence of 100 µM Oxo-M. Cells were then lysed and fractionated as described in the Materials and Methods. (A) Equivalent aliquots (30 µg protein) of subcellular fractions P_1 (crude plasma membrane fraction) and V_1 ('light' membrane fraction) were electrophoresed, transferred to nitrocellulose membranes, and then blotted for EAAT3, actin, and early endosomal antigen 1 (EAA1), as detailed in the Materials and Methods. Blots shown are representative of those obtained in 5 independent experiments. (B) Densitometric analysis of Western blots. Results shown are the density of each fraction divided by that of the appropriate loading control (actin for P_1; EAA1 for V_1; means ± SEM of 5 independent experiments). (C) [^{3}H]QNB binding in P_1 and V_1 fractions was determined; results shown are the means ± SEM of 5 independent experiments and are expressed as fmol bound / mg of protein. $p<0.05$ different from density observed in the absence of Oxo-M under isotonic (#) or hypotonic (##) conditions (by repeated measures ANOVA followed by a Bonferonni multiple comparisons test).

in these subcellular fractions or in cell lysates. To determine whether exposure of SH-SY5Y cells to a hypoosmotic medium resulted in a global disruption of trafficking events, the ability of Oxo-M to induce the redistribution of mAChRs from the plasma membrane to endosomal fractions was monitored. Exposure of the cells to Oxo-M for 10 min resulted in an increase in [^{3}H]QNB binding (~200% of control) in the V_1 fractions that was similar in magnitude under both isotonic and hypotonic conditions (Fig. 4.12C).

Discussion

In addition to its role as a major neurotransmitter glutamate also functions as a quantitatively important organic osmolyte within the CNS. Thus mechanisms that regulate its concentration within the extracellular space are of physiological importance under both isotonic and hypoosmotic conditions. In the present study we have demonstrated that, under isotonic conditions, agonist occupancy of mAChRs leads to a rapid and sustained increase in glutamate uptake (monitored as D-aspartate) into SH-SY5Y neuroblastoma cells. Activation of LPA and endothelin receptors also increased D-aspartate uptake, but to a lesser extent. In C_6 glioma cells, activation of several phosphoinositide-linked receptors has previously been demonstrated to facilitate D-aspartate uptake (Najimi et al., 2002; Fournier et al., 2004; Najimi et al., 2005). In this context, it may be relevant that of the GPCRs tested in SH-SY5Y neuroblastoma, only the mAChR couples to phosphoinositide hydrolysis in this cell line (Heacock et al., 2006a).

D-aspartate uptake into SH-SY5Y cells was mediated via a high affinity, NaCl-dependent, saturable transport system that could be blocked by LβBA, a potent inhibitor of the neuron-specific glutamate transporter, EAAT3. Although three subtypes of the glutamate transporter (EAAT1-3) have been reported to be present in SH-SY5Y cells (Sala et al., 2005), only immunoreactivity to the EAAT3 subtype was detectable in cell lysates in the present study. This observation, along with the potent inhibition of D-aspartate uptake by LβBA and the ability of cysteine to readily compete with D-aspartate for transport (a characteristic of the EAAT3 subtype:

Zerangue and Kavanaugh, 1996), strongly suggests that the primary mediator of D-aspartate uptake in SH-SY5Y cells is EAAT3. In quiescent SH-SY5Y cells, the EAAT3 transporter is concentrated in an intracellular compartment, as previously reported for other cell types (Fournier et al., 2004; Beart and O'Shea, 2007). However, upon mAChR activation in SH-SY5Y cells, a redistribution of the transporter to the plasma membrane occurs (Fig. 4.12A,B). It appears likely that a major component of Oxo-M-mediated increase in D-aspartate uptake in SH-SY5Y cells reflects this appearance of EAAT3 at the plasma membrane, although the possibility that receptor activation increases the intrinsic activity of the transporter cannot be excluded.

In SH-SY5Y cells, the activation of mAChRs elicits a number of biochemical consequences including the activation of kinases, mobilization of intra- and extracellular Ca^{2+} and the re-arrangement of the cytoskeleton (Cioffi and Fisher, 1990; Linseman et al., 1999; Linseman et al., 2000). The present results suggest that the activation of multiple signaling pathways is required for mAChR-mediated regulation of D-aspartate transport. For example, a role for PKC is indicated from the ability of BIM, a PKC inhibitor, to partially block Oxo-M-stimulated D-aspartate uptake (Fig. 4.8). Our results differ from those obtained for C_6 glioma cells in which little or no effect of PKC inhibitors on receptor-simulated EAAT3 activity was observed (Sims et al., 2000; Najimi et al., 2002; Najimi et al., 2005). Based upon the ability of wortmannin to inhibit both basal- and mAChR-mediated D-aspartate transport, PI3K also appears to play a role in the regulation of EAAT3 activity in SH-SY5Y cells (Fig. 4.10), an observation consistent with some, but not all, reports of

receptor-stimulated EAAT3 trafficking and activity (Sims et al., 2000; Najimi et al., 2002; Krizman-Genda et al., 2005; Najimi et al., 2005). One aspect of receptor-mediated EAAT3 regulation that has previously received little attention is that of its dependence on Ca^{2+} availability. The present data strongly suggest that the mAChR-mediated influx of extracellular Ca^{2+} is a major determinant of the magnitude of mAChR-mediated increases in D-aspartate uptake. Influx of extracellular Ca^{2+} may serve to activate downstream effectors such as Ca^{2+} / calmodulin-dependent protein kinase and / or the Ca^{2+}-sensitive forms of PKC. The importance of both PKC and Ca^{2+} in regulation of EAAT3 activity in SH-SY5Y cells is evident from the observation that Ca^{2+} depletion, in conjunction with PKC inhibition, essentially abolishes mAChR-stimulated D-aspartate uptake (Fig. 4.9). These findings are consistent with results recently obtained for C_6 glioma cells in which a sustained entry of Ca^{2+}, elicited independently of receptor activation, resulted in a PKC-dependent increase in both the cell-surface expression and activity of EAAT3 (Murphy et al., 2009). Optimal D-aspartate transport in SH-SY5Y cells under both basal- and Oxo-M-stimulated conditions also requires the maintenance of cytoskeletal integrity (Fig. 4.11). Thus it is possible that these signaling pathways (Ca^{2+}, PKC and PI3K) may act in concert to regulate cytoskeletal-dependent trafficking of EAAT3. Consistent with this possibility, a role for PKC, PI3K and the actin cytoskeleton has been proposed for dopamine receptor-mediated translocation of Na^{+}-K^{+}-ATPase from endosomes to the plasma membrane (Bertorello and Sznajder, 2005).

Previous studies have indicated that, during episodes of hypoosmotic stress, organic osmolyte homeostasis is regulated not only via osmolyte efflux, but also at the level of osmolyte re-uptake (Olson and Martinho, 2006; Foster et al., 2009). Consistent with this possibility, a major finding from the present study is that mAChR-mediated uptake of D-aspartate (and to a lesser extent, basal uptake) is significantly attenuated when SH-SY5Y cells are subjected to hypoosmotic stress. The observation that ET-1-mediated increases in D-aspartate uptake are similarly attenuated by hyposmolarity in C6 glioma cells indicates that osmotic regulation of glutamate transport may be a general, rather than cell-specific, phenomenon. The reductions in D-aspartate uptake in SH-SY5Y cells could be fully reversed upon re-exposure of the cells to an isotonic medium and were elicited by relatively modest reductions in osmolarity (≥ 16%). Inhibition of D-aspartate uptake under hypotonic conditions reflected a reduction in V_{max} for transport, whereas the K_m value was unaltered. The uptake of D-aspartate exhibited similar characteristics in terms of agonist concentration dependence, requirements for PKC and PI3K activity and the need for an intact cytoskeleton under both isotonic and hypotonic conditions. However, two distinct osmosensitive features of D-aspartate uptake are apparent. The first is that under hypoosmotic conditions, D-aspartate uptake is largely independent of extracellular Ca^{2+}. Thus the addition of the Ca^{2+} ionophore, ionomycin, did not significantly increase uptake under hypotonic conditions whereas a robust increase in D-aspartate uptake was apparent under isotonic conditions (Fig. 4.7A,B). Similarly, removal of extracellular Ca^{2+} had little or no effect on mAChR-stimulated D-aspartate uptake under hypoosmotic conditions whereas a marked

inhibition was observed under isotonic conditions (Fig. 4.9A,B). The mechanism whereby the Ca^{2+}-dependent component of mAChR-stimulated D-aspartate uptake is lost under conditions of hyposmolarity remains to be determined. However, this inability of Ca^{2+} to regulate D-aspartate uptake reflects the osmotic disruption of an event *distal* to mAChR activation since hyposmolarity did not attenuate stimulated phosphoinositide hydrolysis, an event proximal to mAChR activation and dependent on a sustained influx of extracellular Ca^{2+} (Fig. 4.2B; Fisher et al., 1989). The second feature of EAAT3 regulation that differs under hypoosmotic conditions is that of it's trafficking. Under hypoosmotic conditions, EAAT3 transporters accumulate at the plasma membrane, with little or no regulation of trafficking following mAChR activation (Fig. 4.12A,B). These observations were unexpected given (1) that the rate of basal D-aspartate uptake is attenuated by a reduction in osmolarity and (2) that although reduced in magnitude, activation of mAChRs still elicits an increase in uptake under hypoosmotic conditions. One possible explanation for the divergence between transporter trafficking and activity under hypotonic conditions is that EAAT3 that accumulates in the plasma membrane is either not correctly inserted and / or has a diminished intrinsic activity. Alternatively, hypotonicity may induce a translocation of the transporter into a vesicular fraction that associates with, but is not integral to, the plasma membrane. However, altered EAAT3 trafficking does not appear to reflect a global disruption of membrane trafficking events in SH-SY5Y cells since Oxo-M-mediated internalization of mAChRs was unaffected by changes in osmolarity (Fig. 4.12C).

The observed osmosensitive regulation of EAAT3-mediated glutamate uptake *in vitro* could potentially have physiological consequences in the intact brain. In this context, evidence for cholinergic regulation of glutamate concentrations in brain has been reported (Higley et al., 2009). mAChR activation may allow EAAT3-containing neurons to maintain or reduce local extracellular glutamate concentrations during small reductions in osmolarity, whereas during more pronounced reductions in osmolarity, depletion of intracellular glutamate would occur. In addition, EAAT3 has been demonstrated to mediate cysteine uptake (Aoyama et al., 2006), the rate limiting step in glutathione synthesis. Glutathione is depleted in hyponatremic brains (Clark et al., 1996), an observation that could potentially be explained by osmosensitive inhibition of EAAT3 activity. However, the physiological relevance of tonicity-mediated changes in basal or mAChR-mediated glutamate uptake remains to be directly evaluated *in vivo*.

In summary, the present results indicate that the activation of mAChRs results in a pronounced stimulation of EAAT3 transporter-mediated D-aspartate uptake into SH-SY5Y cells, under conditions of either isotonicity or mild hyposmolarity. In contrast, mAChR activation results in an inhibition of the re-uptake of the more benign osmolyte, taurine (Foster et al., 2009). As osmolarity is further decreased, the ability of mAChR activation to facilitate the uptake of D-aspartate, while still evident, is progressively attenuated thereby providing additional evidence that, in response to hypoosmotic stress, cells preferentially release organic osmolytes while retaining their complement of inorganic osmolytes (Foster et al., 2008).

Chapter 5

Discussion

Regulation of brain volume is critical to the maintenance of proper neural function during conditions that induce cellular swelling. Hypotonic swelling, as observed during the commonly encountered clinical disorder hyponatremia, can cause neurological side effects such as nausea, seizures, and coma, while overly-rapid treatment can lead to adverse effects such as pontine myelinosis. Understanding the mechanisms whereby brain cell volume is regulated is critical to understanding and concurrently treating pathologies in which brain cell swelling occurs. The primary mechanism whereby brain volume is maintained in the face of hypotonic swelling is through the release of various osmolytes which, as a direct consequence of their release, bring with them osmotically-obligated water. Although brain cells have the intrinsic ability to release osmolytes in response to hyposmotic swelling, this process can be facilitated by the activation of certain GPCRs. These receptors not only increase the magnitude of osmolyte release, but also decrease the osmotic threshold at which osmolyte release occurs. *In vitro*, GPCR activation can induce the efflux of both inorganic, and organic osmolytes, to a similar magnitude (in terms of % osmolyte release), and with a similar osmolarity-

dependence. However, *in vivo* data suggest that inorganic osmolytes, such as K^+ and Cl^-, are relatively retained (10-15% loss) in the brains of chronically hyponatremic rats, when compared to organic osmolytes such as taurine (>95% loss). Although specificity of osmolyte loss has important ramifications due to differing consequences of the release / depletion of various osmolytes from the brain, the mechanisms whereby such regulation occurs have not been previously identified. Because osmolyte loss is a result of both osmolyte release and concurrent osmolyte uptake, I hypothesized that GPCR-mediated regulation of osmolyte uptake is key in determining the specificity of osmolyte loss. Here I will summarize the results of my studies and discuss the implications of these findings with regards to brain volume regulatory mechanisms.

In Chapter 2 the ability of GPCRs, and in particular mAChRs, to modulate both the efflux and influx of K^+ from SH-SY5Y cells was evaluated using the K^+ radiotracer $^{86}Rb^+$. Under isotonic conditions, both $^{86}Rb^+$ efflux and influx were stimulated by administration of Oxo-M. Both $^{86}Rb^+$ efflux and, to a lesser extent, $^{86}Rb^+$ influx, were demonstrated to be osmolarity-dependent, in that both basal- and receptor-mediated fluxes were potentiated as extracellular osmolarity was reduced. Furthermore, Oxo-M-stimulated $^{86}Rb^+$ efflux and influx were observed to have analogous signaling requirements as both were inhibited by removal of intracellular Ca^{2+} and / or inhibition of PKC. Despite these similarities, the two fluxes were determined to be mediated via distinct mechanisms, with influx occurring primarily via the Na^+/K^+ ATPase and NKCC transporters, and efflux demonstrating sensitivity to broad spectrum K^+ channel inhibitors (Fig. 5.1A). Measurement of unidirectional

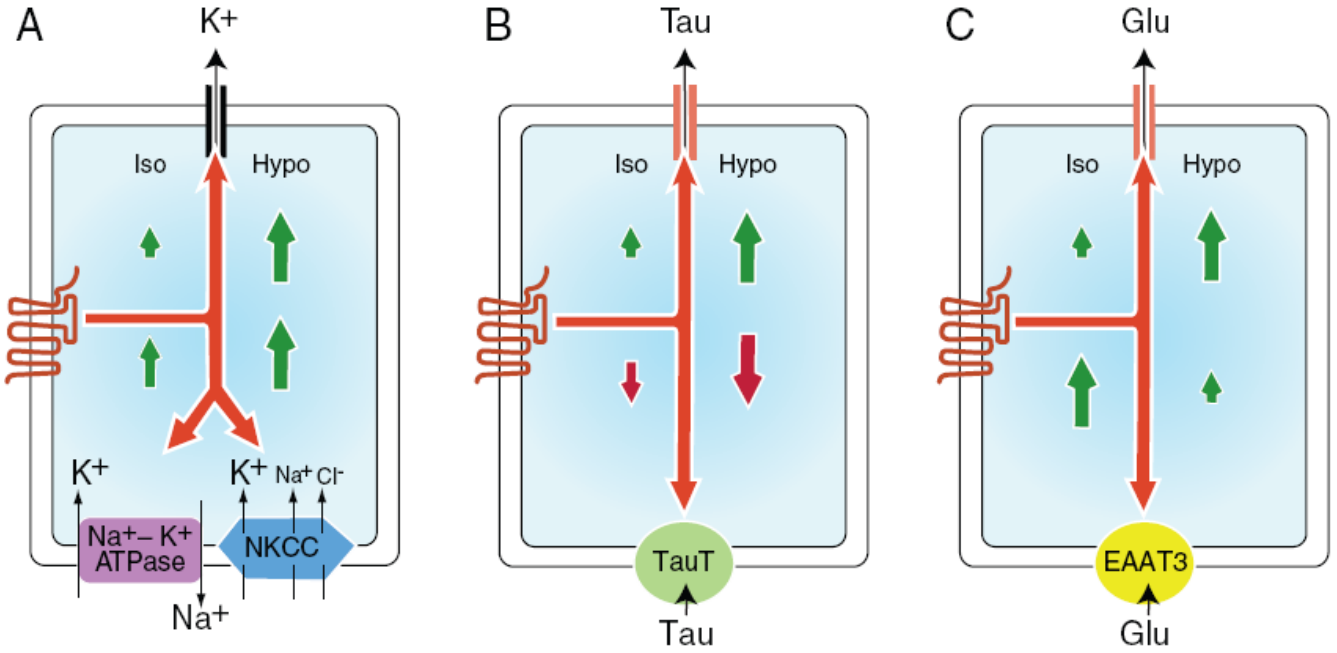

Figure 5.1 Diagrammatic representation of the capacity of GPCRs present on SH-SY5Y cells to regulate the efflux and uptake of K^+, taurine, or glutamate under isotonic or hypotonic conditions. Although only a limited efflux of osmolytes is observed following the activation of GPCRs under isotonic (Iso) conditions, the efflux of all three osmolytes is is markedly increased by hypotonicity (Hypo; as indicated by the relative size of the arrows). The efflux of K^+ is mediated primarily via K^+ channel(s) wherease taurine (Tau) and glutamate (Glu) are released via the VSOAC. K^+ uptake (mediated primarily by the Na^+/K^+-ATPase and NKCC transporters) is facilitated by GPCR activation under isotonic conditions, and this is further enhanced by hyposmolarity. In contrast, under isotonic conditions GPCR activation results in an inhibition of Tau uptake, which is mediated primarily via the taurine transporter (TauT). This inhibition of TauT is further enhanced under hyposmotic conditions. Glutamate uptake, which is mediated by the excitatory amino acid transporter 3 (EAAT3), is markedly increased by GPCR activation under isotonic conditions. However, the stimulation of Glu uptake is progressively inhibited as osmolarity is reduced.

$^{86}Rb^+$ fluxes does not permit a direct comparison of their relative effects on intracellular K^+. To circumvent this problem, the intracellular K^+ concentration of cells exposed to various conditions was measured directly with an ion-specific electrode. Such K^+ measurements demonstrated that activation of mAChRs under either isotonic, or mild (-15%) hypotonic conditions, resulted in little or no net change in intracellular K^+, unless K^+ influx was concurrently inhibited with ouabain or furosemide. This indicates that, under these conditions, Oxo-M-stimulated K^+ efflux is countered by an agonist-stimulated K^+ influx of a similar magnitude. Exposure of the cells to Oxo-M under more hyposmotic conditions (-30%) resulted in a decrease in intracellular K^+ concentrations, a result that reflects the relatively robust osmosensitivity of Oxo-M-stimulated K^+ efflux when compared to K^+ influx. Over the past 45 years it has become evident that astrocytes play a vital role in regulating extracellular K^+ clearance and neural excitability (Walz, 2000). Similar to SH-SY5Y cells, when cultures of primary astrocytes were exposed to ligands that activate PAR-1 or lysophospholipid receptors that had been previously shown to induce taurine efflux in an osmosensitive manner, both $^{86}Rb^+$ influx and efflux were stimulated. Taken collectively, these findings suggest that GPCR-stimulated K^+ influx could provide a mechanism whereby K^+ is selectively retained by the brain during hyposmotic volume regulation, as is observed in the brains of chronically hyponatremic rats *in vivo*.

Maintenance of intracellular K^+ concentrations within strict limits during cell volume regulation could have beneficial consequences, because the loss of K^+ can adversely impact membrane potential and ionic strength. Whereas the data

obtained provide a possible explanation as to how brain cells maintain relatively constant K^+ concentrations during hyposmotically-induced RVD, it does raise the question of why such a cumbersome mechanism would exist. It would seem redundant to release K^+, only to immediately expend energy in the form of ATP to re-accumulate the recently released K^+. Hypotonicity, however, is not the only condition in which K^+ release and uptake occur concurrently. Under resting isotonic conditions, intracellular K^+ concentrations are maintained at ~150mM in neural tissues via an equilibrium that exists between the uptake of K^+ (mediated primarily by the Na^+/K^+ ATPase), and the loss of K^+ (through leak channels), that has been dubbed the 'leak / pump' pathway. This pathway is crucial to maintaining intracellular volume, membrane potential, and overall cellular homeostasis. Most proteins are located intracellularly, possess a negative net charge, and are essentially impermeable to the membrane. In the absence of other factors, these conditions would cause a Gibbs-Donnan equilibrium in which the osmolarity of the intracellular compartment would be greater than that of the extracellular fluid. This would create hydrostatic pressure, concurrent water influx and eventually, cell lysis. However, by virtue of Na^+/K^+ ATPase-mediated Na^+ efflux, and the relative lack of Na^+-permeant leak channels, Na^+ is made effectively impermeant to the membrane. In such a manner, the cell creates a highly concentrated, positively charged, and effectively membrane-impermeant, extracellular Na^+ pool which acts to offset the negatively charged intracellular proteins and allows an equilibrium, designated the 'double Donnan equilibrium', to be reached. This double Donnan equilibrium is advantageous in that the osmolarity of both the intracellular and extracellular

compartments is equal, and thus allows volume homeostasis (Hoffmann et al., 2009). In summary, the cell releases and accumulates K^+ under isotonic conditions, a mechanism that seems redundant in terms of maintaining intracellular K^+ concentrations, but is necessary to regulate Na^+ transport in such a way as to maintain cellular homeostasis. Accordingly, the utility of increasing both K^+ efflux and influx under hypotonic conditions may not be energy efficient with regards to K^+ homeostasis, but may advantageously alter the transport of other molecules such as Na^+ and Cl^-.

The capacity of GPCRs to affect taurine loss from SH-SY5Y cells via modulation of taurine influx was examined in Chapter 3. In contrast to the results reported for K^+, both hypotonicity and mAChR activation lead to a significant decrease in taurine uptake, a mechanism that when combined with increased taurine efflux would further potentiate loss of taurine from these cells (Fig. 5.1B). Taurine uptake into SH-SY5Y neuroblastoma cells was mediated via a Na^+-dependent, high affinity, saturable transport system with pharmacological characteristics similar to that previously reported for the cloned taurine transporters (TauT-1 and TauT-2). While both Oxo-M-stimulated taurine influx and efflux are inhibited by removal of extracellular Ca^{2+}, only taurine efflux demonstrated sensitivity to inhibition of PKC. This result suggests that, under hypotonic conditions, mAChR-mediated taurine influx and efflux are activated by distinct signaling pathways. In addition, while hypotonicity- and mAChR-mediated stimulation of taurine efflux is relatively transient, with the rate of taurine efflux in the first 10 min being far greater than that observed thereafter, reductions in the rate of taurine influx persist for at

least 1 hour. For receptor activation to induce enduring changes in taurine uptake, that receptor must either be resistant to ligand-induced desensitization, or alternatively, mediate its effects on taurine uptake via mechanisms with long half-lives. Such considerations are likely to be of little importance in determining the ability of a receptor to transiently activate taurine efflux, suggesting that receptors, even if they are equal in terms of their ability to stimulate taurine efflux, may display different capacities in their ability to persistently regulate taurine uptake. When considering the possible importance of taurine uptake to taurine homeostasis, one must consider the basal level of TauT activity in a particular tissue. Under isotonic conditions and in the presence of a saturating taurine concentration, TauT has the potential to replenish ~8% of the taurine content of SH-SY5Y cells within 1 h. Under hyposmotic conditions, this value decreases to ~4%. Since SH-SY5Y cells maximally release approximately 20% of their taurine content in response to mAChR activation under hypotonic conditions, the contribution of TauT to the taurine content of cells is likely to be significant, and increasingly so as time progresses given the persitence of taurine uptake. Such results were not restricted to SH-SY5Y cells, since both hypotonicity and addition of thrombin or S1P were able to reduce taurine influx in cultures of primary astrocytes. Accordingly, regulation of taurine uptake may represent a mechanism whereby taurine homeostasis can be significantly modulated in brain cells, even after cell volume correction may already be complete.

An unexpected finding was that inclusion of 10 μM DCPIB, a molecule that has been previously demonstrated to selectively inhibit swelling-induced Cl^- and organic osmolyte efflux, completely blocked both Oxo-M-stimulated taurine efflux

and influx. Dose-response analysis revealed that DCPIB blocked both basal- and mAChR-induce taurine efflux with an IC_{50} ~3 μM. However, although DCPIB blocked basal taurine uptake with a similar potency, it did not have any effect on mAChR-stimulated taurine uptake until it was administered at doses >6 μM. Surprisingly, given the postulated site of DCPIB action as an anion channel blocker, it was found that inclusion of 10 μM DCPIB was also able to completely attenuate both mAChR-mediated $^{86}Rb^+$ efflux and influx. Because VSOAC, the channel through which volume-sensitive Cl^- and organic osmolyte efflux is thought to occur, has yet to be molecularly identified, it is impossible to verify if this channel represents the site at which DCPIB exerts its primary effects in SH-SY5Y cells. Given the diverse array of transporters and channels involved in taurine and K^+ fluxes (i.e. Na^+/K^+ ATPase, NKCC, KCC, K^+ channels, TauT, VSOAC), and the inability of DCPIB to alter basal K^+ or taurine fluxes under isotonic conditions, it would seem unlikely that DCPIB is a direct inhibitor at each transporter or channel.

Several models of DCPIB-mediated inhibition of osmolyte fluxes can be postulated. One possibility is that a permissive level of VSOAC activation is necessary for concurrent changes in K^+ fluxes and taurine influx to occur. Accordingly, only reductions of VSOAC activity below a certain permissive rate, would result in an inhibition of K^+ fluxes and taurine influx. Physical interactions have been reported between the Na^+/K^+ ATPase and the VSOAC candidate phospholemman, as well as between the NKCC and the band 3 anion exchanger, another proposed mediator of VSOAC current (Crambert and Geering, 2003; Guizouarn et al., 2004). These observations raise the possibility that the effects of

DCPIB could be mediated via a physical interaction between the VSOAC and the other volume-sensitive channels and transporters. Alternatively, DCPIB may not exert its effects at the site of transport, but alternatively at the volume sensor itself. Consequently, inhibition of this volume sensor (whose identity remains unknown), would effectively inhibit all volume-sensitive osmolyte fluxes from occurring. However, as mentioned above, volume-sensitive basal- and receptor-mediated taurine influx appear to display differential sensitivities to DCPIB, which would seem to argue against the above hypothesis. Although the mechanism whereby DCPIB inhibits these osmolyte fluxes remains an enigma, these data suggest that the influx and efflux of distinct osmolytes may occur in a much more integrated fashion than previously appreciated. Accordingly, future identification of the target of DCPIB, and the mechanism whereby DCPIB inhibits various osmolyte fluxes, could potentially contribute significantly to our knowledge of cell volume regulatory processes.

The data in Chapters 2 and 3 demonstrate that mAChR activation under hypotonic conditions stimulated the uptake of the inorganic osmolyte K^+, while inhibition of the uptake of the organic osmolyte taurine was observed. However, from these two studies it is not clear whether osmosensitive K^+ and taurine fluxes are representative of other inorganic and organic osmolytes respectively. Although an increase in the extracellular concentration of a relatively inert organic osmolyte such as taurine might have minimal cellular consequences, a similar increase in the concentration of the organic osmolyte glutamate would be potentially deleterious due to its ability to induce ‘spreading depression’ and, at higher concentrations

excitotoxicity. Accordingly, in Chapter 4 the ability of osmolarity and mAChR activation to regulate glutamate uptake was evaluated.

Under isotonic conditions, activation of mAChRs resulted in a significant increase in glutamate uptake (monitored with ^{3}H-D-aspartate), and a redistribution of the excitatory amino acid transporter 3 (EAAT3) to the plasma membrane. However, hypotonicity attenuated the ability of mAChR activation to stimulate glutamate uptake and disrupted EAAT3 cellular trafficking (Fig. 5.1C). The mAChR-mediated increase in D-aspartate uptake was dependent on cytoskeletal integrity, availability of intracellular Ca^{2+}, and the activities of PKC and PI3K. In contrast, a dependence upon extracellular Ca^{2+} was only observed under hypotonic conditions. These results indicate that GPCR-mediated changes in glutamate influx display both similarities, and differences, in the characteristics of K^{+} or taurine influx. Accordingly, mAChR activation stimulates glutamate uptake (as it does K^{+}), while hypotonicity decreases mAChR-activated glutamate uptake (as is the case for taurine influx; Fig. 5.1). Such a process may permit cellular glutamate to be conserved following small reductions in osmolarity wheras it is depleted upon exposure to more hyposmotic conditions.

Pharmacological and immunoblotting analysis detailed in Chapter 4 suggest that EAAT3 is the primary subtype of glutamate transporter present in SH-SY5Y cells and that EAAT3 trafficking to the plasma membrane is stimulated upon activation of mAChRs. This is consistent with previous findings that several GPCRs, many of which couple to phosphoinositide hydrolysis, stimulate D-aspartate uptake and EAAT3 translocation under isotonic conditions in C6 glioma cells (which

exclusively express the EAAT3 subtype; Najimi et al., 2002; Fournier et al., 2004; Najimi et al., 2005). In both SH-SY5Y and C6 glioma cells, receptor-stimulated D-aspartate uptake was significantly attenuated by hypotonicity. Although hypotonicity has previously been demonstrated to alter the function of several proteins (Lang et al., 1998a), as well as affect cytoskeletal integrity and exocytosis in various preparations (Franco et al., 2008; Vazquez-Juarez et al., 2008b), the effects of hypotonicity on protein trafficking have received little attention. The exact mechanism(s) whereby hypotonicity alters the trafficking of EAAT3, but not of the mAChR, remains to be elucidated, but these data raise the possibility that hypotonicity selectively affects protein trafficking in a previously unrecognized manner.

Neither receptor activation, nor hypotonicity, had any effect on D-aspartate uptake in cultures of primary astrocytes, which are known to predominantly express EAAT1 and EAAT2 (Beart and O'Shea, 2007), raising the possibility that the EAAT3 subtype is uniquely regulated by hypotonicity and receptor activation. EAAT3 expression is limited to neurons which, in the CNS, are generally thought to be outnumbered by glia 10:1, although recent studies have suggested that this ratio may be lower than previously reported (Azevedo et al., 2009; Hilgetag and Barbas, 2009). In addition to its neuron-specific expression, EAAT3 can be found primarily in the intracellular compartments of quiescent cells (Fournier et al., 2004). These characteristics suggest that EAAT3 may not play a predominant role in the clearance of glutamate from whole brain under quiescent conditions, however, its role would be enhanced upon GPCR stimulation. Accordingly, neuron-specific glutamate uptake in

the hyponatremic brain may be initially triggered by the release of a GPCR ligand, which in turn activates EAAT3 activity. However, hypotonicity itself will increasingly inhibit EAAT3 function as osmolarity is reduced. The net result of these two processes is that during phases of relatively minor reductions in osmolarity, glutamate will be effectively cleared from the synaptic cleft. However, under conditions of more pronounced hyposmolarity, it is anticipated that glutamate will accumulate extracellularly. Further studies are needed to determine the magnitude, and osmosensitivity, of these opposing mechanisms on glutamate uptake *in vivo*.

In addition to glutamate uptake, EAAT3 has also been demonstrated to be the primary mediator of cysteine uptake in neural cells. Intracellular cysteine can be incorporated into many different proteins including, but not limited to, the anti-oxidant glutathione, and cysteine uptake is thought to be the rate-limiting step for glutathione synthesis in neural cells (Aoyama et al., 2006). Induction of chronic hyponatremia (plasma Na^+ decreased by ~25%) has been reported to decrease brain glutathione content in both rats *in vivo* and human cell lines *in vitro*. This glutathione loss was found to occur in the absence of any identifiable glutathione efflux, suggesting that reduced synthesis and / or increased degradation were the likely causes (Clark et al., 1996). Our results suggest that hypotonicity of this magnitude may reduce EAAT3-mediated cysteine uptake and therefore contribute to the observed reduced glutathione synthesis.

Homogenous populations of SH-SY5Y cells provided a convenient model system, with defined receptor populations and extensively characterized signal transduction pathways, in which to assess of the ability of sudden reductions in

osmolarity to regulate osmolyte fluxes. Key findings were then verified in cultures of primary rat astrocytes, where practical, so as to broaden the scope of the findings. Measurements of K^+, taurine, and glutamate influx, and efflux, predict that K^+ will be comparatively retained, taurine severely depleted, and glutamate retained at small reductions in osmolarity with depletion escalating as osmolarity is reduced (Fig. 5.1). Confidence in this model is bolstered by the agreement between these predictions and measurements of osmolytes lost from both cultures of astrocytes exposed to hypotonic conditions (Kimelberg et al., 1990; Olson, 1999), and the brains of chronically hyponatremic rats (Fig. 1.3). In both of these systems, K^+ is largely retained (<10% loss), whereas taurine depletion is severe (>85%). In cultures of primary astrocytes, either acute (1 hr) or chronic (24 hr) exposure to hypotonicity (-20%), had little to no effect on glutamate content (Olson, 1999), while further reductions in osmolarity (-35%) induced significant glutamate loss (~50%) within 1 hr (Kimelberg et al., 1990). Intermediate glutamate loss (50%) was also observed from chronically hyponatremic rat brains (Fig. 1.3). While the osmolyte flux data detailed in this thesis are in agreement with these studies, the *in vitro* model employed during osmolyte flux measurements does have several caveats that must be considered when attempting to translate the findings from this system to *in vivo* models of hyponatremia.

It is clear that glial cells make the major contributions to volume regulation in the CNS *in vivo* (Kimelberg, 1995), in large part due to their relative abundance. Whether or not neurons swell, and consequently undergo RVD, *in vivo* has been debated in the literature. Freshly isolated hippocampal neurons have been reported

to lack volume regulation (Andrew et al., 1997; Aitken et al., 1998), and it has been suggested that loss of taurine during tissue preparation may account for this discrepancy. Thus, RVD is observed in hippocampal slices only when they are supplemented with taurine (Kreisman and Olson, 2003). However, unlike glia, pyramidal neurons in slices of cerebral cortex appear to be relatively resistant to swelling when exposed to a sudden 25% reduction in osmolarity, regardless of whether taurine is present or not (Andrew et al., 2007). One possible explanation for the observation that pyramidal neurons do not swell under hypotonic conditions, is that these neurons may have membranes that demonstrate decreased water permeability due to the fluidity or lipid composition of their plasma membrane, as is the case with cells lining the stomach and the distal renal tubule cells (Lavelle et al., 1997; Krylov et al., 2001; Tian et al., 2004). However, previous reports have demonstrated that *in vivo* reductions in plasma osmolarity of 20% result in taurine release from cerebellar Purkinje neurons in the absence of a measurable increase in cell volume (Nagelhus et al., 1993). This has raised the possibility that neurons may regulate their volume on a second to second basis and thus actively extrude osmolyte-associated water as fast as water enters. When hippocampal slices are subjected to a gradual hyposmotic challenge (~2.5 mOsM/min), cell swelling is not observed, even under conditions of 50% hypotonicity (Franco et al., 2000; Pasantes-Morales et al., 2000b). Under this condition, which is known as isovolumetric regulation, a slow efflux of osmolytes counters potential increases in cell volume. Different cells express different capacities to perform isovolumetric regulation, and it has been reported that C6 glioma cells are relatively deficient in this regard, as

extremely slow rates of osmolarity reduction (0.4 mOsM/min) are required for volume to remain constant (Lohr and Yohe, 2000). This could suggest that certain cells (i.e. pyramidal neurons), possess an enhanced capability to regulate their volume, and may explain why these cells are relatively resistant to swelling. Receptor-mediated regulation of osmolyte fluxes may facilitate the resistance to cell swelling observed in pyramidal neurons, in a manner similar to that reported for Müller glia cells. In retinal slices, these cells do not swell upon exposure to hypotonicity unless their purinergic receptors are inhibited, indicating the presence of receptor-mediated mechanisms that enhance osmolyte release and concurrent volume regulation (Wurm et al., 2010). Taken together, these findings suggest that pyramidal neurons may be able to release osmolytes and counteract cell swelling in such a manner that significant swelling is not observed, whereas the surrounding astrocytes swell because they possess less efficient cell volume regulatory mechanisms. Thus, it is apparent that cell volume regulatory mechanisms occur in most (if not all) cells in the brain and act to maintain brain volume under conditions of hyposmotic stress.

The brain possesses a complexity of structure that cannot be recapitulated *in vitro*. While the vast majority of studies in the literature examine the ability of cells to extrude intracellular osmolytes, this process represents only the first step in osmolyte loss from the brain. Upon release into the interstitial fluid (ISF), osmolytes must then leave the brain and enter the systemic circulation if they are to affect brain volume. The processes whereby osmolytes exit the ISF are just as important to brain volume regulation as the mechanisms regulating the loss of osmolytes from

brain cells, however the processes whereby the former occurs remain poorly understood. During the initial onset of hyponatremia a rapid and transient loss of NaCl from the ISF is observed, however Na^+ transport across the blood brain barrier is unchanged (Melton et al., 1987). This has lead to the hypothesis that the route of exit for the NaCl is via the CSF, although evidence in support of this is mostly indirect. Further studies on how osmolytes, both inorganic and organic, are translocated from the ISF to the systemic circulation are necessary to further elucidate the mechanisms whereby brain volume is regulated.

Throughout the studies described in Chapters 2-4, sudden reductions in extracellular osmolarity were utilized to initiate osmolyte fluxes. While this experimental paradigm provides robust cellular responses *in vitro*, such a model does not recapitulate the diseased state, as the onset of hyponatremia *in vivo* is likely to occur in a gradual manner. While gradual reductions in buffer osmolarity of different magnitudes have been found to activate distinct populations of K^+ channels in C6 glioma cells (Ordaz et al., 2004), there is little evidence to date that reductions in osmolarity, of a similar magnitude, activate different mechanisms when they are administered gradually or suddenly. Nonetheless, future elucidation of basal- and GPCR-regulated osmolyte influx and efflux during conditions of gradual osmotic change would strengthen the case for the importance of these mechanisms in the hyponatremic brain.

In vitro, supra-physiological reductions in osmolarity are necessary to induce osmolyte fluxes from neural cells under basal conditions. However, activation of certain GPCRs has been demonstrated to reduce the osmotic threshold at which

osmolyte release occurs, raising the possibility that GPCR activation is necessary for efficient cell volume regulation in neural cells *in vivo*. Most studies examining the role of receptor-mediated volume regulation involve the addition of exogenous ligand. However, hypotonicity itself promotes the release of several such ligands. Even in the absence of exogenous ligand, blockade of either purinergic or AMPA / kainate receptors in the substantia nigra *in vivo*, prevents hyposmotically-induced taurine release as measured by microdialysis (Morales et al., 2007), indicating that, in this system, endogenous receptor activation facilitates osmolyte efflux. Similarly, either genetic deletion of P_{2Y} receptors, or their inhibition by addition of antagonists, can significantly increase hypotonically-induced cell swelling in Müller glia from retinal slices (Wurm et al., 2010). These studies demonstrate the potential importance of endogenously stimulated receptor activation to neural cell volume regulation. However, further *in vivo* studies are needed to elucidate the functional importance of such mechanisms.

GPCR-mediated volume regulation could potentially display a large variation amongst different brain regions, as it would require a proximal relationship of the cells that release ligands and those that possess volume-regulating receptors that are activated by the ligands. Although tonic receptor activation could affect cell volume regulation, hypotonically-induced ligand release could provide an additional mechanism whereby cell volume is regulated. Further complexity could be introduced by receptors that negatively regulate cell volume processes, as has been reported with the adenosine A_{2B} receptor (Pierson et al., 2007). In such a manner, receptor regulation may allow for ‘fine-tuning’ of osmolyte homeostasis in that

different receptors could act in a co-ordinated manner to both enhance and inhibit volume-regulatory mechanisms. Cells in different brain regions also may express different subtypes of osmosensitive channels and transporters, or express the same subtypes at different levels, which could potentially affect their ability to respond to changes in cell volume. To date, our understanding of brain volume regulation *in vivo* has predominately been performed at the whole brain level, however, future analysis of hyponatremia in different brain regions may reveal that volume regulation is not homogenously regulated across the brain. Analysis of the impact of different receptors *in vivo*, however is complicated by the diversity of receptors that have been found to mediate volume-sensitive osmolyte fluxes and the potential for redundancy in the system. Overcoming these hurdles in the future to examine the importance of these GPCRs, and of hypotonicity-induced ligand release, to affect cell volume regulatory processes *in vivo*, is critical to furthering our understanding of brain volume regulation.

Furthermore, the role of GPCRs in both aiding the normalization of brain volume, and in the promotion of undesired pathological consequences, must be considered. In the CNS, thrombin, at low concentrations (50 pM to 100 nM), can exert neuroprotective effects, preventing hypoglycemic- or hypoxic- induced damage, whereas higher concentrations of the protease can cause cell death and disruption of the blood brain barrier (Xi et al., 2003). Addition of thrombin facilitates the osmosensitive release of glutamate and taurine from astrocytoma, neuroblastoma, and cortical astrocyte preparations (Cheema et al., 2005; Cheema et al., 2007; Ramos-Mandujano et al., 2007; Cheema and Fisher, 2008). The effects

of thrombin are mediated by the PAR-1 receptor subtype in all three tissues and require subnanomolar or low nM concentrations of thrombin for half-maximal activation of osmolyte release, concentrations that are consistent with the neuroprotective properties of the protease. However, even low doses of thrombin can exacerbate brain injury caused by ischemia, iron, 6-hydroxydopamine, or tissue plasminogen activator (Figueroa et al., 1998; Hua et al., 2003b; Nakamura et al., 2005; Cannon et al., 2007). While the mechanism of this enhanced injury induced by low concentrations of thrombin remains to be established, an enhanced efflux of glutamate via thrombin-induced VSOAC activity may play a role, particularly in the case of ischemia, where cell swelling may accentuate this process.

In addition to potentiating the release of excitotoxic glutamate under hyposmotic conditions, receptor activation will also induce the loss of several other neuroactive compounds including GABA, glycine, aspartate, and N-acetyl-aspartate. Release of these compounds, and subsequent receptor activation, could negatively impact cellular function. The potential for osmolyte-mediated receptor activation is accentuated by the fact that under hyposmotic conditions the volume of the extracellular space will shrink as a result of cell swelling. Under resting conditions the extracellular space is predicted to occupy approximately 20% of the brain volume (Sykova, 2004). However, as water osmotically enters the cell during cell swelling, one would expect the extracellular space volume to decrease. Accordingly, increased ligand release, in the presence of a contracted extracellular space, has the potential to adversely elevate extracellular neurotransmitter concentrations. Under such conditions even relatively inert molecules, such as taurine, may exert

negative effects by activating $GABA_A$, $GABA_B$, NMDA, and glycine receptors. Therefore, GPCR-mediated organic osmolyte fluxes may represent a 'double-edged sword' in that the beneficial effects of volume regulation may offset by the negative consequences of neuroactive osmolyte release.

Activation of certain GPCRs regulates both the efflux and influx of various osmolytes under hypotonic conditions *in vitro*. These mechanisms may allow cells to regulate their volume while depleting themselves of relatively inert osmolytes such as taurine, and selectively retaining others (K^+, glutamate; Fig 5.1). Further studies are needed to determine the physiological, and pathophysiological, significance of such receptor-mediated volume regulation *in vivo* as well as to further elucidate the mechanisms whereby these processes are mediated. By broadening our knowledge of how brain cells regulate their volume, we improve our ability to design targeted therapies to treat diseases in which brain cell volume homeostasis is disrupted.

Appendix

Prostanoid Receptors Regulate the Volume-Sensitive Efflux of Osmolytes from Murine Fibroblasts via a Cyclic AMP-Dependent Mechanism

Summary

The ability of prostanoid receptors to regulate the volume-dependent efflux of the organic osmolyte, taurine, from murine fibroblasts (L cells) via an adenosine 3':5'-cyclic monophosphate (cAMP)-dependent mechanism has been examined. Incubation of L cells under hypoosmotic conditions resulted in a time-dependent efflux of taurine, the threshold of release occurring at 250mOsM. Addition of prostaglandin E_1 (PGE_1) potently (EC_{50}=2.5 nM) enhanced the volume-dependent efflux of taurine at all time points examined and increased the threshold for osmolyte release to 290 mOsM. Maximal PGE_1 stimulation (250-300% of basal) of taurine release was observed at 250 mOsM. Of the PGE analogs tested, only the EP_2-selective agonist, butaprost, was able to enhance taurine efflux. Inclusion of 1,9-dideoxyfoskolin (DDF), 5-nitro-2-(3-phenylpropylamino benzoic acid (NPPB) or 4-[(2-Butyl-6,7-dicloro-2-cyclopentyl-2,3-dihydro-1-oxo-1H-inden-5-yl)oxy]-butanoic acid (DCPIB) blocked the ability of PGE_1 to enhance taurine release, indicating the

mediation of a volume-sensitive organic osmolyte and anion channel (VSOAC). The ability of PGE_1 to increase osmolyte release from L cells was mimicked by the addition of agents that inhibit cAMP breakdown, directly activate adenylyl cyclase or are cell-permeant analogs of cAMP. Taurine release elicited by either PGE_1 or 8-CPT-cAMP was attenuated by >70% in L cells that had been stably transfected with a mutant regulatory subunit of cAMP-dependent protein kinase (PKA). PGE_1-stimulation of taurine efflux was not attenuated by either depletion of intracellular calcium or inhibition of protein kinase C. The results indicate that activation of prostanoid receptors on murine fibroblasts enhances osmolyte release via a cAMP and PKA-dependent mechanism.

Introduction

The ability of cells to respond to alterations in their osmotic environment is of fundamental importance to their survival. In response to hypoosmotic stress, cells initially swell with a magnitude proportional to the reduction in osmolarity, but this is followed by a recovery process of regulatory volume decrease in which osmolytes (K^+, Cl^- and 'compatible' organic osmolytes) are extruded and cell volume normalized following the exit of obligated water (McManus et al., 1995; Pasantes-Morales et al., 2000; Pasantes-Morales et al., 2002). Polyols, methylamines and amino acids are the principal organic osmolytes utilized by eukaryotic cells. Of these, the amino acid taurine appears to be ideally suited because of its metabolic inertness and abundance (Lambert 2004). The extrusion of Cl^- and organic osmolytes such as taurine is thought to occur via a volume-sensitive organic osmolyte and anion channel (VSOAC) which is primarily permeable to Cl^- but is impermeable to cations (Nilius et al., 1997; Lang et al., 1998; Kimelberg, 2000; Nilius and Droogmans, 2003). The efflux of Cl^- generates an outwardly rectifying current known as $I_{Cl,swell}$. Regulatory volume decrease, organic osmolyte efflux and $I_{Cl,swell}$ can be blocked not only by non-selective Cl^- channel inhibitors such as DDF or NPPB but also by the highly selective inhibitor of VSOAC, DCPIB (Decher et al., 2001; Abdullaev et al., 2006). In addition to cell volume regulation, several other cell functions have been attributed to VSOACs. These include modulation of electrical activity, cell cycle progression, cell proliferation, apoptosis and metabolic regulation (for reviews, see Nilius et al., 1997; Lang et al., 1998; Stutzin and Hoffman, 2006).

When measured *in vitro*, the efflux of organic osmolytes is relatively insensitive to hypoosmotic stress, often requiring substantial (non-physiological) reductions in osmolarity before a significant efflux of osmolytes occurs. This observation, along with previous reports that swelling-induced osmolyte release can be enhanced by Ca^{2+} ionophores, phorbol esters or agents known to elevate cAMP (Strange et al., 1993; Novak et al., 2000; Moran et al., 2001), raises the possibility that, *in vivo*, the activity of VSOAC may be regulated by the activity of G-protein coupled receptors (GPCRs). In this context, we and others have recently identified a number of Ca^{2+}-mobilizing GPCRs that, when activated, enhance the volume-sensitive efflux of osmolytes from both neural and non-neural cells: P2Y purinergic receptors in rat primary astrocytes (Mongin and Kimelberg, 2002, 2005), H_1 histamine receptors in HeLa cells (Falktoft and Lambert, 2004), m_3 muscarinic cholinergic (mAChR), lysophosphatidic acid and sphingosine 1-phosphate receptors in human SH-SY5Y neuroblastoma (Loveday et al., 2003; Heacock et al., 2004, 2006) and the protease-activated receptor-1 (PAR-1) in myoblasts and human 1321N1 astrocytoma (Manopoulos et al., 1997; Cheema et al., 2005). Receptor activation has been demonstrated to facilitate the ability of the cells to release osmolytes under conditions of very limited reductions in osmolarity (5-10%) via a mechanism that appears to involve intracellular Ca^{2+} and protein kinase C (PKC) activity.

A major signal transduction pathway utilized by a large number of GPCRs is the activation of adenylyl cyclase with the concomitant formation of cAMP. In this context, the addition of forskolin, a direct activator of adenylyl cyclase, has been

reported to increase osmolyte release in some, but not all, tissues (Strange et al., 1993; Manopoulos et al., 1997; Moran et al., 2001). Electrophysiological recordings indicate that cAMP can also increase $I_{Cl,swell}$ although inhibitory effects of the cyclic nucleotide have also been reported (Carpenter and Peers, 1997; Du and Sorota, 1997; Nagasaki et al., 2000; Shimizu et al., 2000). Although these results indicate a potential role for cAMP in osmoregulation, the ability of endogenously expressed adenylyl cyclase-linked receptors to regulate osmolyte efflux has not been systematically examined. In the present study, we have evaluated the ability of prostanoid receptors present in murine L fibroblasts to regulate osmolyte efflux under conditions of hypoosmotic stress. These cells are known to possess prostanoid receptors that robustly couple to adenylyl cyclase and PKA activation (Maganiello and Vaughn, 1972; Uhler and Abou-Chebl, 1992). The results indicate that activation of prostanoid receptors (principally of the EP_2 subtype) facilitates a volume-dependent increase in osmolyte release that is mediated via a VSOAC. The stimulatory effect of PGE_1 on taurine efflux can be mimicked by agents that elevate intracellular cAMP and are attenuated in an L cell line that exhibits reduced PKA activity. Moreover, in contrast to the responses elicited by agonist occupancy of Ca^{2+}-mobilizing receptors, osmolyte efflux triggered by prostanoid receptor activation is independent of both intracellular Ca^{2+} and PKC.

Materials and Methods

Materials. [1,2-^{3}H]Taurine (1.1 TBq/mmol) was obtained from Amersham Biosciences (Piscataway, NJ). [γ-]^{32}P-ATP (111 TBq/mmol) was from Perkin Elmer Life Sciences, (Boston, MA). NPPB, sphingosine 1-phosphate (S1P), forskolin, thrombin, 8-CPT-cAMP, 8-bromo-cAMP, pepstatin A, phenylmethylsulfonyl fluoride, 1,10-phenanthroline, dithiothreitol, cAMP, ATP and Kemptide (Leu-Arg-Arg-Ala-Ser-Leu-Gly) were purchased from Sigma-Aldrich (St. Louis, MO). Prostaglandin E_1 was from Biomol (Plymouth Meeting, PA). DDF, Gö 6983 and IBMX were obtained from Calbiochem (San Diego, CA). DCPIB was purchased from Tocris Biosciences (Ellisville, MO). Guanidinethyl sulfonate was obtained from Toronto Chemicals (Toronto, ON). Fura-2/acetoxymethyl ester (Fura-2/AM) was from Molecular Probes (Eugene, OR). Butaprost (free acid), 17-phenyl trinor PGE_2 and sulprostone were obtained from Cayman Chemical (Ann Arbor, MI). Dulbecco's modified Eagle medium (DMEM), genetecin (G418), 50x penicillin/streptomycin and horse serum were obtained from Invitrogen (Carlsbad, CA). Universol was obtained from ICN biomedical (Urora, OH).

Cell culture conditions.

Murine Ltk^{-} fibroblasts (L cells: passage numbers 5-19) and RAB-10 cells (an L cell derived cell line which exhibits reduced PKA activity: Uhler and Abou-Chebl, 1992, passage numbers 5-11) were grown in tissue culture flasks (75 cm^2/250 ml) in 20 ml of DMEM supplemented with 10% (v/v) horse serum and 1% penicillin/streptomycin.

For the RAB-10 cells, 750 μg/ml of G418 (geneticin) was included. The osmolarity of the medium was 330-340mOsM. Cells were grown at 37°C in a humidified atmosphere containing 5% CO_2. The medium was aspirated and the cells detached from the flask with a tryspin-versene mixture (Biowhittaker, MD). Cells were then resuspended in DMEM/10% horse serum with penicillin/streptomycin and subcultured into 35 mm, six-well culture plates at a density of 250-300,000 cells/well for 4-5 days. Cells that had reached 95-100% confluency were routinely used.

Measurement of taurine efflux.

Osmolyte efflux from L cells and RAB-10 cells was monitored essentially as previously described for SH-SY5Y neuroblastoma (Heacock et al., 2004, 2006). In brief, L cells were prelabeled overnight with 18.5 KBq/ml of [^{3}H]taurine at 37^{o}C. Under these conditions, approximately 90-95% of the added radiolabel was taken up into the cells. Uptake of radiolabel into L cells was time-dependent ($t_{1/2}$ ~3h), temperature-sensitive (inhibited >98% by lowering the temperature to 4^{o}C) and was inhibited >70% by inclusion of a 500 μM guanidinethyl sulfonate, an inhibitor of the taurine uptake transporter (Lambert, 2004). After prelabeling, the cells were washed with 2 x 2 ml of isotonic Buffer A (142 mM NaCl, 5.6 mM KCl, 2.2 mM $CaCl_2$, 3.6 mM $NaHCO_3$, 1mM $MgCl_2$ and 30 mM HEPES, pH 7.4, 1 mg/ml D-glucose; approx. 335 mOsM). Cells were then allowed to incubate in 2 ml of hypotonic buffer A (295-195 mOsM; rendered hypotonic by a reduction in NaCl concentration) in the absence or presence of PGE_1. In some experiments, buffer A was made hypertonic (370 mOsM) by the addition of NaCl. Osmolarities of buffer A were monitored by means

of an Osmette precision osmometer (PS Precision Systems, Sudbury, MA). At times indicated, aliquots (200 μl) of the extracellular medium were removed and radioactivity determined after the addition of 5 ml of Universol scintillation fluid. The reactions were terminated by rapid aspiration of the buffer and cells lysed by the addition of 2 ml of ice-cold 6% (wt/vol) trichloroacetic acid. Taurine efflux was calculated as a fractional release, i.e., the radioactivity released in the extracellular media as a percentage of the total radioactivity present initially in the cells. The latter was calculated as the sum of radioactivity recovered in the extracellular medium and that remaining in the lysate at the end of the assay (Novak et al., 1999). "Basal" release of taurine is defined as that which occurs at a specified osmolarity in the absence of agonists or cyclic AMP analogs.

Measurement of PKA activity.

PKA activity was determined essentially as described by Uhler and McKnight (1987). L cells or RAB-10 cells were harvested by detaching the cells from the flasks with a rubber policeman and centrifugation at 5,000 x g for 5 min. Cell pellets were then resuspended in homogenization buffer (10 mM $NaPO_4$ buffer (pH 7.0), 1 mM EDTA, 1 mM dithiothreitol, 1 mM iodoacetic acid, 0.1 mM phenylmethysufonyl fluoride, 1 mM 1,10-phenanthroline, 1 mM pepstatin A and 250 mM sucrose), sonicated (6 x 1 s) and protein concentrations adjusted to 1 mg/ml. Assays (50 μl final volume) were conducted for 5 min at 30^{o} C and contained (final concentrations) 100 μM ATP (250 dpm/ pmol), 5 mM Mg acetate, 15 μM Kemptide, 250 μM IBMX, 5 mM dithiothreitol, 2.5 mM NaF and 10 mM Tris-HCl (pH 7.4). When included in the assay, the

concentration of cAMP was 5 μM. The phosphorylation of Kemptide was determined by spotting 25 μl of the incubation mixture on Whatman PE81 phosphocellulose filter papers (2 x 2 cm) and washing them with 4 x 200 ml of 10 mM orthophosphoric acid. After a final wash in 95% ethanol, individual filters were allowed to dry at room temperature and radioactivity determined after the addition of 5 ml of Universol scintillation fluid.

Measurement of Cytoplasmic Calcium Concentrations.

Cytoplasmic free calcium concentrations, $[Ca^{2+}]_i$, were determined in suspensions of L cells after preloading cells with the Ca^{2+} indicator, fura-2 AM, as previously described (Fisher et al., 1989). The fluorometer used was a Shimadzu RF-5301PC spectrofluorometer (Shimadzu Scientific Instruments, Columbia, MD).

Data analysis.

Experiments were performed in triplicate and repeated at least three times. Except where stated otherwise, values quoted are given as means ± S.E.M. for the number (n) of independent experiments indicated. A two-tailed Student's t-test (paired or un-paired) was used to evaluate differences between two experimental groups (level of significance, $p<0.05$). One-way or repeated measures Analysis of Variance (ANOVA) followed by Dunnett's multiple comparisons test was used for statistical significance of differences between multiple groups. EC_{50} values were obtained using Prism 4.0a (GraphPad Software, Inc. San Diego, CA).

Results

Osmosensitive efflux of taurine from L cells is enhanced by the addition of PGE_1. When L cells that had been prelabeled with [^{3}H]taurine were exposed to hypotonic buffer (250 mOsM), there was a time-dependent efflux of the radiolabeled amino acid, the initial rate of which (<5 min) exceeded that observed in more prolonged incubations (Fig. A.1). Inclusion of PGE_1 (20 μM) significantly enhanced the rate of release of taurine at all time points examined and increased the magnitude of response to approximately 250-300 % of basal (basal release being that monitored in the absence of an agonist). As a result of these observations, both basal and agonist-stimulated taurine efflux were routinely monitored after 10 min of incubation in subsequent experiments. During the course of the present study, some inter-experimental variability in the magnitude of the basal release of taurine (5.2 ± 2.6% of total, mean $\pm$ SD, n=65) and in the extent of PGE_1-stimulated taurine efflux (274 ± 74% of control, mean $\pm$ SD, n=65) was observed. The addition of PGE_1 resulted in a concentration-dependent stimulation of taurine efflux with an EC_{50} value of 2.5 ± 0.4 nM (n=3) and a Hill coefficient close to unity (1.1 ± 0.2, n=3, Fig. A.2). To determine the subtype of prostanoid receptor coupled to osmolyte efflux, L cells were incubated in the presence of 1 μM concentrations of PGE_1, butaprost (EP_2-selective), 17-phenyl-trinor PGE_2 (EP_1- and EP_3-selective) or sulprostone (EP_3-selective). Of these analogs, only butaprost elicited a significant increase in taurine release (Fig. A.3).

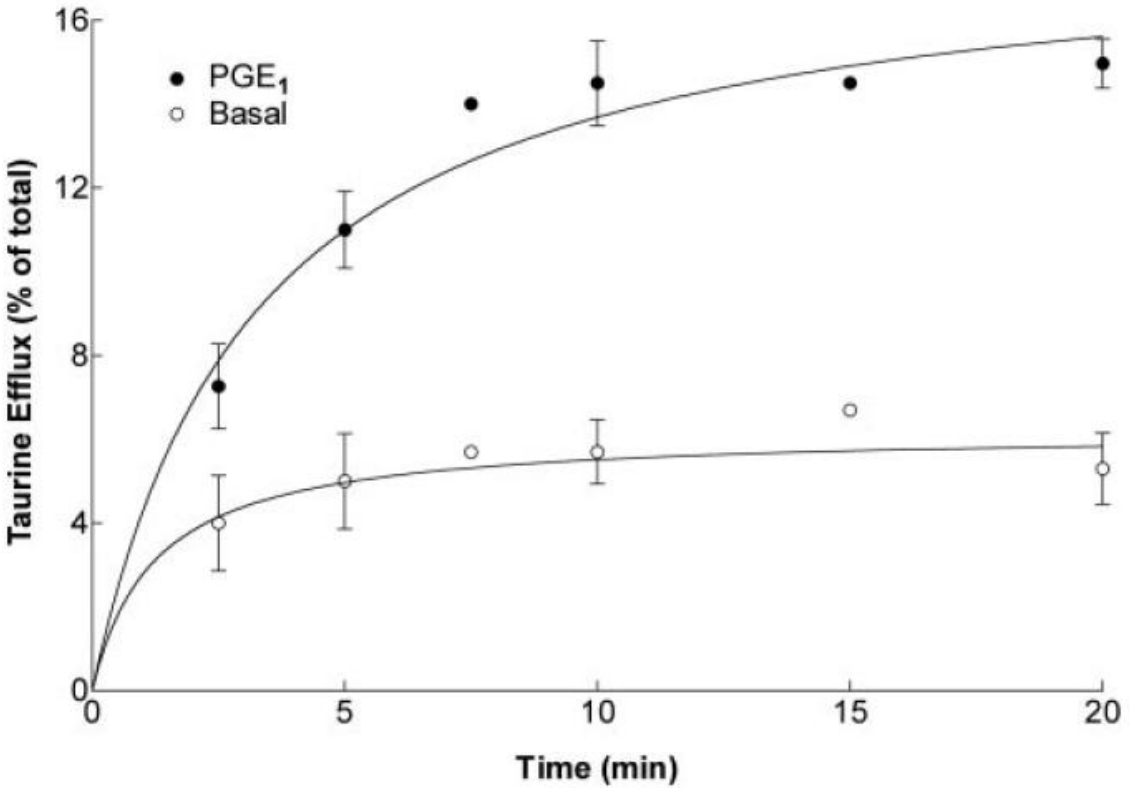

Figure A.1 Kinetics of basal- and PGE_1-stimulated taurine efflux from L cells. L cells that had been prelabeled overnight in the presence of [^{3}H]taurine were washed twice with 2 ml of isotonic buffer A before incubation in 250 mOsM buffer A in the presence or absence of PGE_1 (20 µM). Reactions were terminated at the times indicated and taurine efflux measured. Results are expressed as taurine efflux (percentage of total soluble radioactivity) and (where error bars are shown) are the means ± S.E.M. for three independent experiments, each performed in triplicate. Where errors bars are absent, the result from a single experiment is shown.

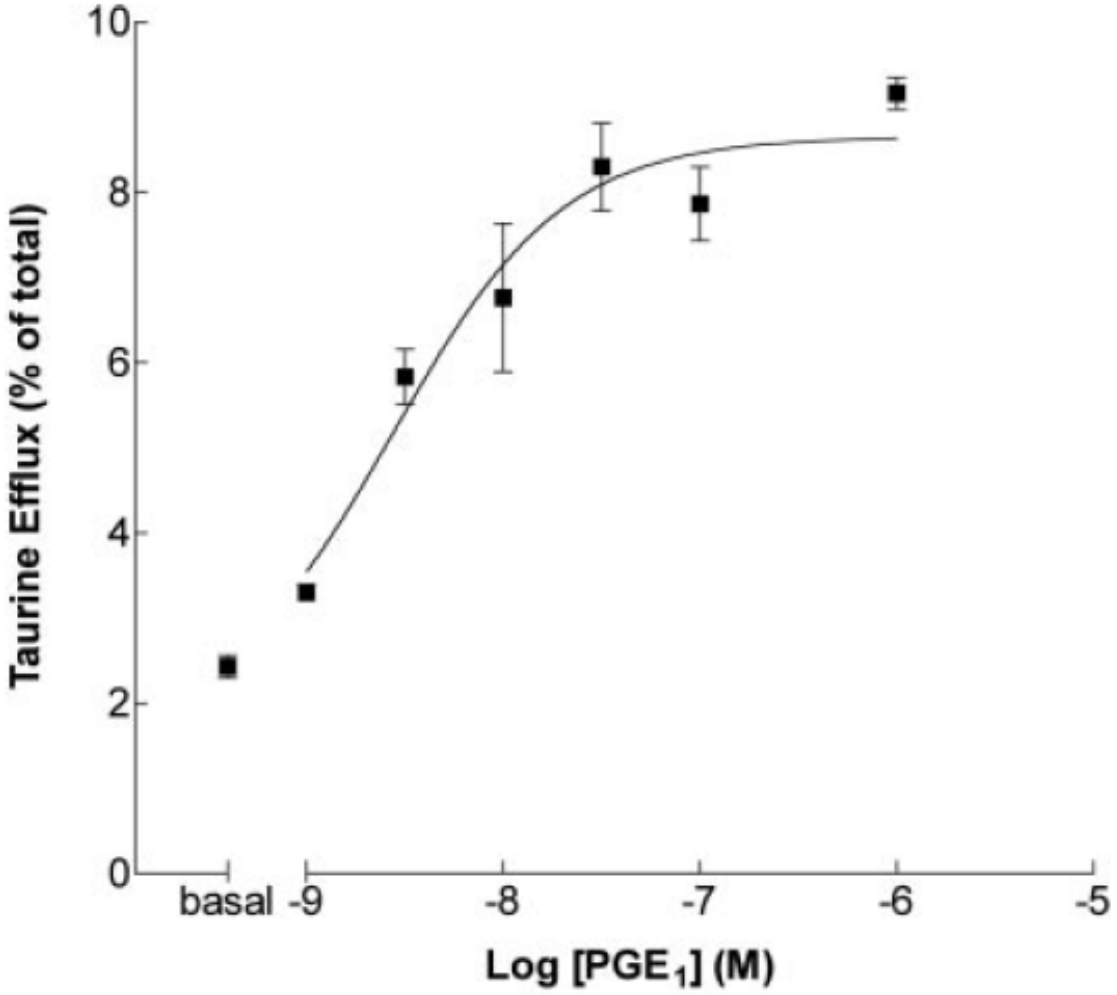

Figure A.2 Dose-response relationships for PGE_1-stimulated taurine efflux. Cells that had been prelabeled with [^{3}H]taurine were washed with isotonic buffer A and then incubated in 250 mOsM buffer in the presence of PGE_1 at the concentrations indicated. Reactions were terminated after 10 min and taurine efflux was monitored. Results are expressed as taurine efflux (percentage of total soluble radioactivity) and are the means ± S.E.M. for triplicate replicates obtained from a single experiment, representative of three conducted. Where error bars are not shown, the S.E.M. fell within the symbol. In the experiment shown, the calculated EC_{50} value for stimulated taurine efflux was 2.0 nM and the Hill coefficient was 0.8.

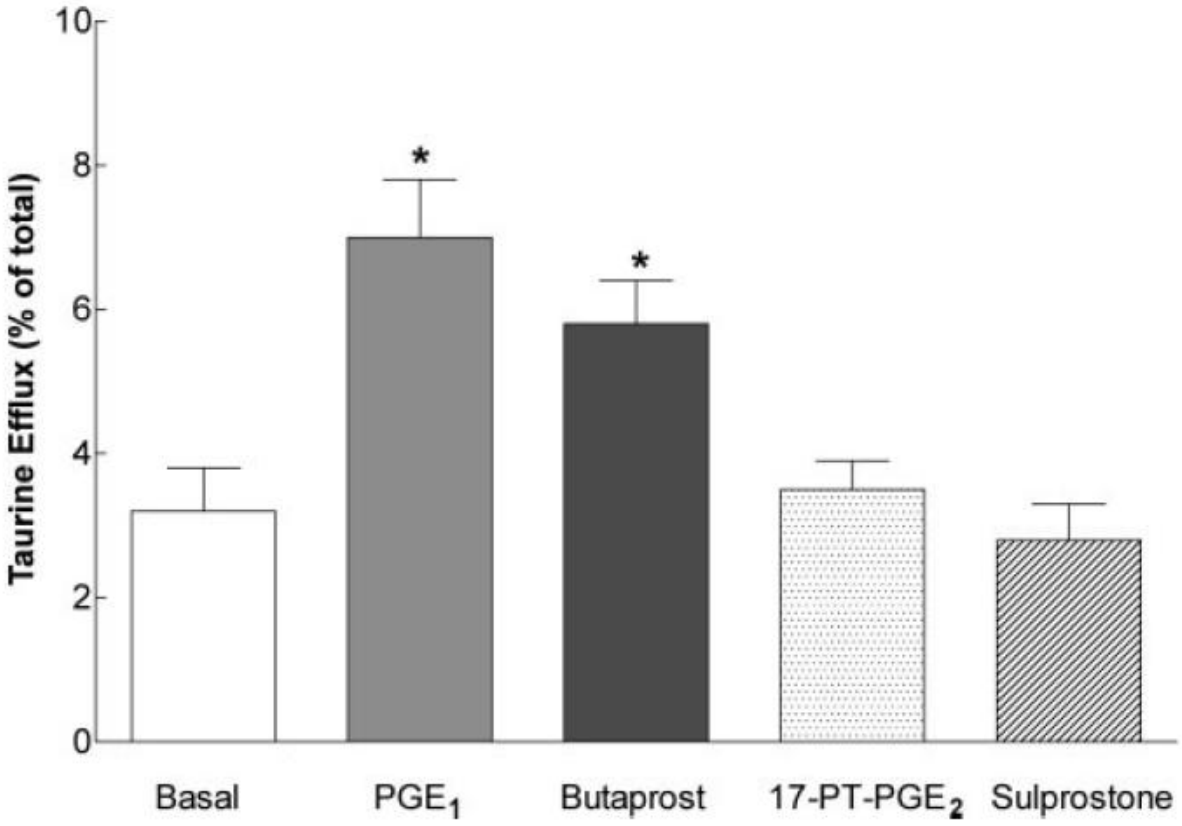

Figure A.3 Butaprost, an EP_2-selective agonist, mimics the ability of PGE_1 to stimulate taurine release. Cells that had been prelabeled with [^{3}H]taurine were washed in isotonic buffer A and then incubated in 250 mOsM buffer in the presence or absence of 1 μM concentrations of PGE_1, butaprost, 17-phenyl trinor PGE_2 (17-PT-PGE_2) or sulprostone. Reactions were terminated after 10 min and taurine efflux was monitored. Results are expressed as taurine efflux (percentage of total soluble radioactivity) and are the means ± S.E.M. for 5 independent experiments, each performed in triplicate. *, different from basal, $p<0.01$ (by paired student's t-test).

The ability of PGE_1 to enhance the volume-sensitive efflux of taurine from L cells is dependent upon osmolarity. The ability of PGE_1 to enhance the release of taurine at different osmolarities was examined. Both basal and PGE_1-stimulated release of taurine were monitored under conditions of isotonicity (335 mOsM: defined by the osmolarity of the DMEM/horse serum medium in which the cells were grown), mild- severe hypotonicity (295-190 mOsM) or mild hypertonicity (370 mOsM). In the series of experiments conducted, the basal release of taurine (i.e. that monitored in the absence of an agonist) was not significantly enhanced until the osmolarity of the buffer had been reduced to 250 mOsM. In contrast, the addition of PGE_1 resulted in a significant increase in taurine efflux under mildly hypotonic conditions (290 mOsM; Fig. A.4). Moreover, as the osmolarity of the buffer was reduced, the ability of PGE_1 to enhance taurine efflux over the basal component was further increased. The maximum enhancement of taurine efflux was observed at an osmolarity of 250 mOsM (350% of basal), but not under either isotonic or mildly hypertonic conditions (Fig. A.4). As a result of these findings, an osmolarity of 250 mOsM was chosen for all subsequent experiments.

PGE_1-mediated efflux of taurine from L cells is mediated via a VSOAC. Previously, we demonstrated that osmolyte release triggered by the activation of mAChRs, PARs or lysophospholipid receptors is mediated via a VSOAC (Heacock et al., 2004,2006; Cheema et al., 2005). To determine whether taurine release elicited by the activation of prostaglandin receptors also occurred via the same channel(s), basal and PGE_1-stimulated taurine efflux were monitored in the

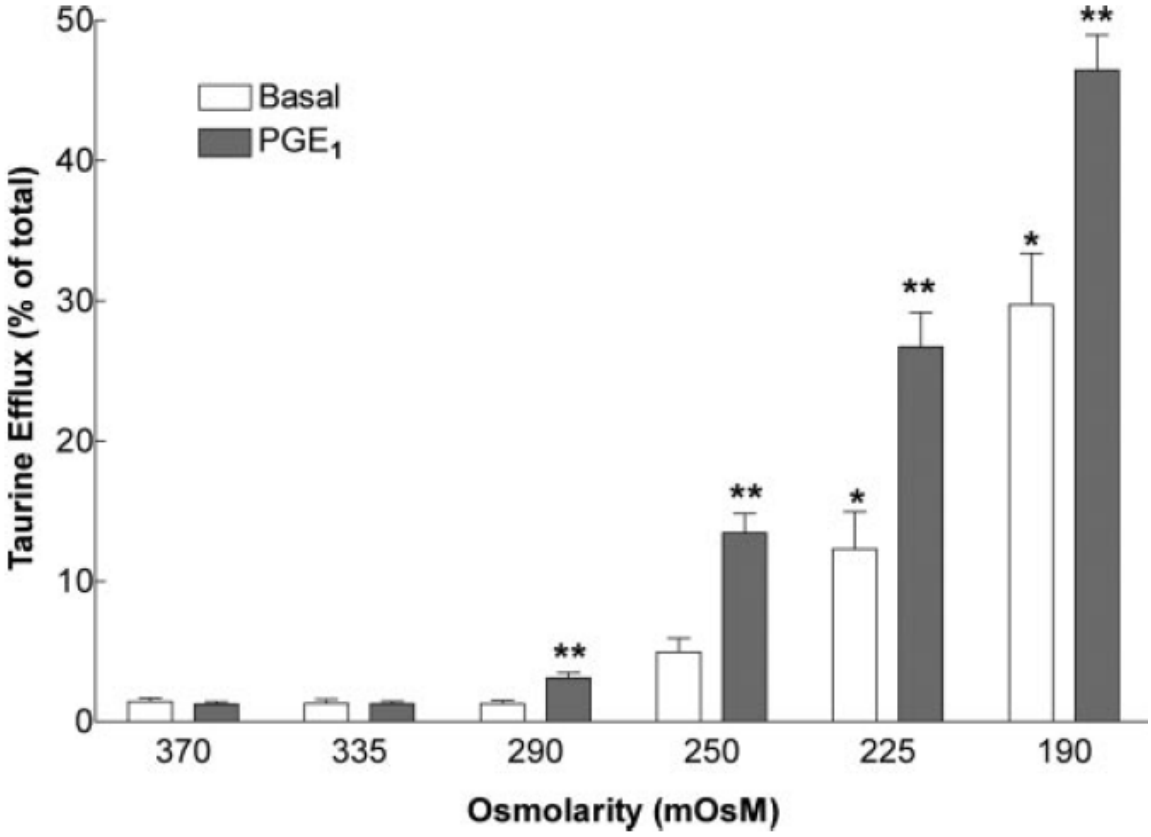

Figure A.4 Basal- and PGE_1-stimulated release of taurine as a function of osmolarity. Cells prelabeled with [^{3}H]taurine were first washed in isotonic buffer A and then incubated for 10 min in buffer A at the osmolarities indicated in the absence (open bars) or presence of 20 µM PGE_1 (solid bars). Reactions were terminated after 10 min and taurine efflux was monitored. Results are expressed as taurine efflux (percentage of total soluble radioactivity) and are the means ± S.E.M. for 4 independent experiments, each performed in triplicate. *, different from taurine release observed in cells incubated in isotonic buffer A (335 mOsM), $p<0.05$ (by one-way ANOVA followed by Dunnett's multiple comparison test). **, different from basal release, $p<0.01$ (by paired student's t-test).

presence of three putative blockers of VSOAC, namely NPPB, DDF and DCPIB. Each of these agents (at concentrations of 100 μM for DDF and NPPB or 30 μM for DCPIB) resulted in a significant inhibition of both basal- and PGE_1-stimulated taurine release (45-62% and 74-90%, respectively, Fig. A.5).

Agents that elevate cAMP concentrations mimic the ability of PGE_1 to enhance taurine efflux. Because PGE_1 is reported to substantially increase cAMP concentrations in L cells (Manganiello and Vaughn, 1972; Uhler and Abou-Chebl, 1992), we examined agents that are known to elevate cAMP concentrations for their ability to increase taurine efflux. Under hypoosmotic conditions (250 mOsM), the addition of 1 mM IBMX, a cAMP phosphodiesterase inhibitor, resulted in an increase in taurine release (174% of basal) that was approximately 65-70% of that elicited by PGE_1 (212% of control). In the combined presence of IBMX and PGE_1, taurine release was less than additive (235% of control:Fig. A.6). Taurine release could also be increased by the addition of 50 μM forskolin, a direct activator of adenylyl cyclase (195% of basal) or alternatively by the addition of 1 mM concentrations of cell permeant analogs of cAMP, namely 8-CPT-cAMP or 8-bromo-cAMP (179-208% of basal, Fig. A.6). When either forskolin or 8-CPT-cAMP were added to incubations that contained PGE_1, no further increase in osmolyte efflux was observed.

PGE_1 stimulation of taurine efflux is mediated via PKA. To investigate whether the stimulation of taurine efflux by PGE_1 and cAMP analogs was mediated via PKA, the ability of PGE_1 or 8-CPT-cAMP to enhance taurine release was monitored in

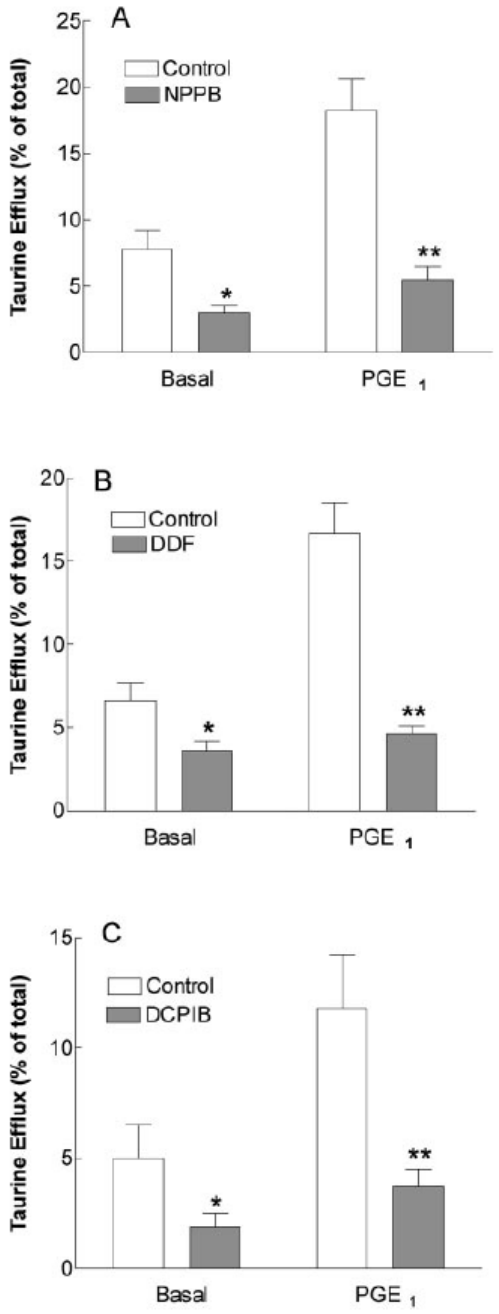

Figure A.5 Inhibition of basal- and PGE_1-stimulated taurine release by anion channel blockers. Cells that had been prelabeled overnight with [^{3}H]taurine were washed in isotonic buffer A and then incubated in hypotonic buffer A (250 mOsM) with either (A) 100 μM NPPB or (B) 100 μM DDF or (C) 30 μM DCPIB in the absence (open bars) or presence (filled bars) of 20 μM PGE1. Reactions were terminated after 10 min and efflux of taurine monitored. Results are expressed as taurine efflux (percentage of total soluble radioactivity) and are the means ± S.E.M. of 6-8 independent experiments, each performed in triplicate. *, different from control basal, $p<0.05$. **, different from PGE_1-stimulated efflux under control conditions, $p<0.03$ (by paired Student's t-test).

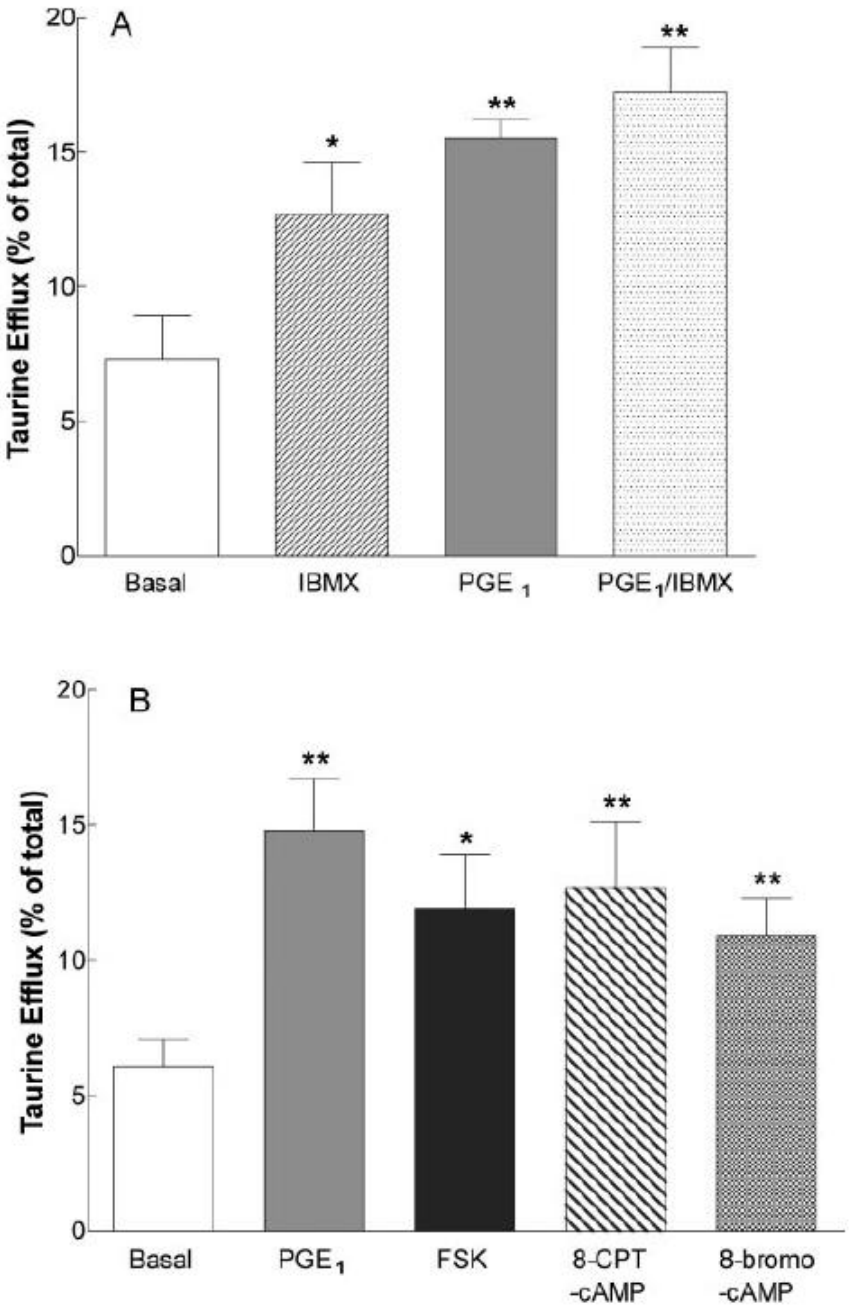

Figure A.6 Agents that increase cAMP concentrations in cells mimic the ability of PGE_1 to enhance taurine release. Cells that had been prelabeled overnight with [^{3}H]taurine were washed in isotonic buffer A and then incubated in hypotonic buffer A (250 mOsM) with either (A) IBMX (1 mM), PGE_1 (20 μM) or both or (B) forskolin (FSK, 50 μM), 8-CPT-cAMP (1 mM) or 8-bromo cAMP (1 mM). Reactions were terminated after 10 min and efflux of taurine monitored. Results are expressed as taurine efflux (percentage of total soluble radioactivity) and are the means ± S.E.M. of 4-6 independent experiments, each performed in triplicate. *, **different from control basal, $p<0.05$, $p<0.01$ (by paired Student's t-test).

L cells and RAB-10 cells. The latter are L cells that have been stably transfected with a mutant regulatory subunit of PKA that renders them less susceptible to activation by cAMP (Uhler and Abou-Chebl, 1992). Preliminary data indicated that the time course of taurine release and sensitivity to osmotic stress were similar in the two cell lines. The ability of either PGE_1 or 8-CPT-cAMP to enhance taurine efflux under hypoosmotic conditions was reduced by <70% in the RAB-10 cells when compared to the untransfected L cells. Thus for L cells, the addition of PGE_1 or 8-CPT-cAMP increased taurine release to 372 $\pm$ 42 and 267 $\pm$ 9% of control, respectively, n=5, whereas the corresponding values for RAB-10 cells were 135 $\pm$ 8 and 127 $\pm$ 10% of control, n=5. In contrast, the ability of either thrombin or sphingosine 1-phosphate to elicit an increase in taurine efflux was not significantly reduced in RAB-10 cells (Fig. A.7). Measurement of PKA activity in extracts of L cells and RAB-10 cells revealed that enzyme activity was reduced by >45% when assayed either in the presence or absence of the cyclic nucleotide (Fig. A.7).

PGE_1 stimulation of taurine efflux is independent of intracellular Ca^{2+} and PKC activity. To date, a common characteristic of those agonists that have been demonstrated to promote the efflux of osmolytes from cells is their ability to elicit increases in $[Ca^{2+}]_i$. Similarly, the addition of PGE_1 to fura-2 loaded L cells also resulted in a 2-3-fold rise in $[Ca^{2+}]_i$. Thrombin addition also elicited an increase in $[Ca^{2+}]_i$ (Fig. A.8). The rise in $[Ca^{2+}]_i$ triggered by the addition of PGE_1 appears to be independent of cAMP formation since neither the addition of forskolin nor 8-CPT-cAMP had any significant effect on $[Ca^{2+}]_i$. Furthermore, no significant increase in

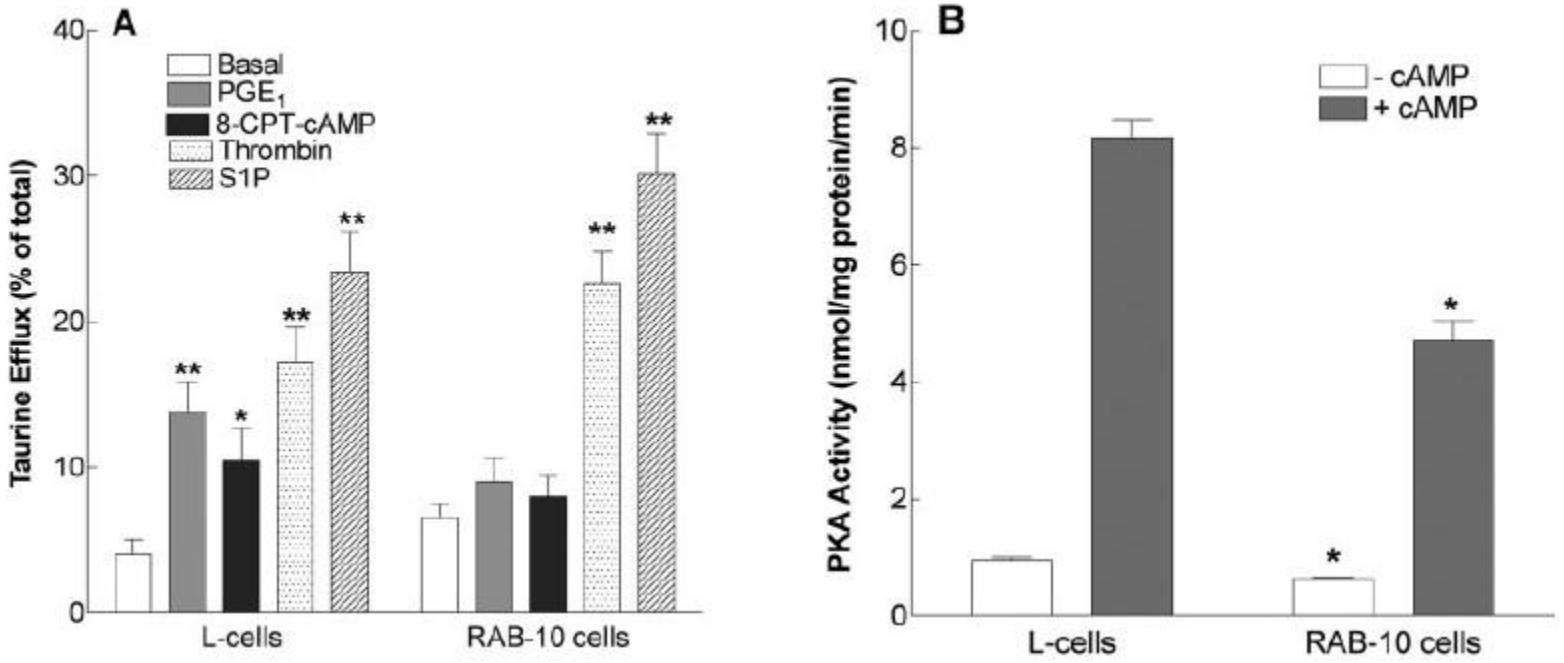

Figure A.7 The ability of PGE_1 or 8-CPT-cAMP to stimulate taurine efflux is attenuated in RAB-10 cells that exhibit a reduced PKA activity. (A) L cells or RAB-10 cells that had been prelabeled overnight with [^{3}H]taurine were washed in isotonic buffer A and then incubated in hypotonic buffer A (250 mOsM) with either PGE_1 (20 μM), 8-CPT-cAMP (1 mM), thrombin (1.25 nM) or sphingosine 1-phosphate (S1P,10 μM). Reactions were terminated after 10 min and efflux of taurine monitored. Results are expressed as taurine efflux (percentage of total soluble radioactivity) and are the means ± S.E.M. for 5 independent experiments, each performed in triplicate. *, **different from control basal, $p<0.03$, $p<0.006$ (by paired Student's t-test). (B) PKA activity was measured in extracts of L- or RAB-10 cells in the absence or presence of 10 μM cAMP. Results shown are means ± S.E.M. for 7 independent experiments, each performed in triplicate. *, different from L cells, $p<0.001$ (by paired Student's t-test).

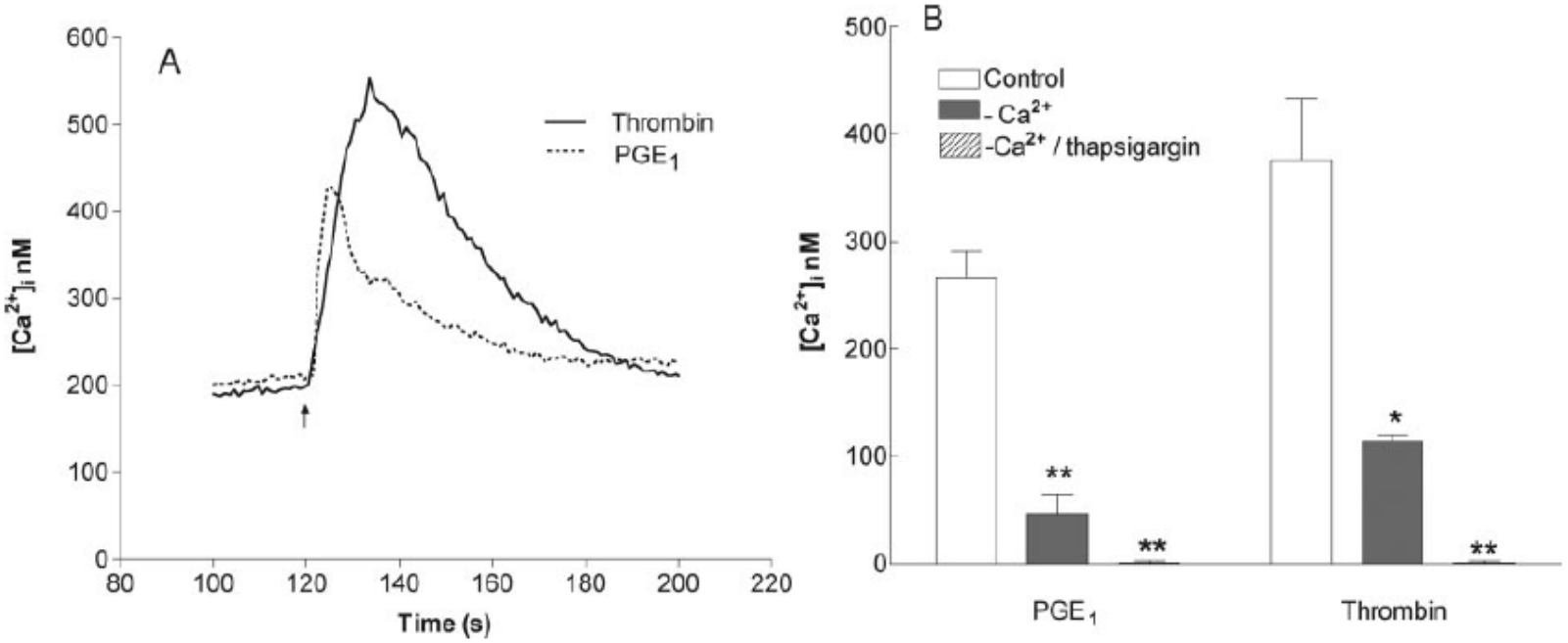

Figure A.8 Addition of PGE_1 or thrombin elicits an increase in cytoplasmic free calcium in L cells. (A) Fura-2/AM loaded cells were first resuspended in 250 mOsM buffer A and then either PGE_1 (20 µM) or thrombin (1.25 nM) added. Changes in $[Ca^{2+}]_i$ were monitored after the addition of the agonists at 120 sec (indicated by the arrow). Traces shown are representative of 15-17 experiments. (B) For each agonist, the maximum increase in $[Ca^{2+}]_i$ was monitored under control conditions (2.2 mM Ca^{2+}; open bars), in the absence of extracellular Ca^{2+} (solid bars) or following pretreatment of the cells for 5 min with 1 µM thapsigargin in the absence of extracellular Ca^{2+}(hatched bars). Results shown are means ± S.E.M. for 15-17 experiments (+Ca^{2+}) or 4 experiments (-Ca^{2+}, -Ca^{2+}/thapsigargin). *, **, different from control, $p<0.05$, $p<0.001$ (by paired Student's *t* test).

$[Ca^{2+}]_i$ was observed in the presence of butaprost (data not shown). The agonist-induced increases in $[Ca^{2+}]_i$ evoked by PGE_1 and thrombin were both markedly attenuated when extracellular Ca^{2+} was omitted (>75%) and abolished following depletion of the intracellular pool of Ca^{2+} with thapsigargin (Fig. A.8).

The ability of PGE_1 to stimulate Ca^{2+} mobilization in L cells was unexpected and prompted us to examine the role, if any, played by Ca^{2+} in basal-, PGE_1- or thrombin- stimulated taurine efflux. Removal of extracellular Ca^{2+} reduced the swelling-induced (basal) release of taurine and that elicited by the addition of either PGE_1 or thrombin, to the same extent, i.e. approximately 30-35%. However, when expressed relative to their controls, PGE_1-stimulated taurine efflux was only minimally reduced by the omission of Ca^{2+} (203 ± 13 and 183 ± 8% of control for PGE_1 in the presence and absence of extracellular Ca^{2+}, respectively, n=10, Fig. A.9). Similarly, thrombin-stimulated taurine efflux was also unaffected by the removal of extracellular Ca^{2+} (276 ± 28 and 289 ± 47% of control, in the presence and absence of Ca^{2+}, respectively, n=6, Fig. A.9). To examine the role of intracellular Ca^{2+}, cells were first preincubated for 5 min in the presence of I μM thapsigargin (in the absence of extracellular Ca^{2+}) to discharge the intracellular Ca^{2+} pools and then challenged with either PGE_1 or thrombin. Under these conditions, PGE_1-stimulated taurine efflux was not significantly reduced (177 ± 6 and 183 ± 8% of control, in the presence and absence of thapsigargin, respectively). Basal release of taurine was also unaffected by depletion of intracellular Ca^{2+}. In contrast, thrombin-stimulated taurine efflux was diminished by
65% following depletion of intracellular Ca^{2+} (167 ± 9% and 289 ± 47% of control, in

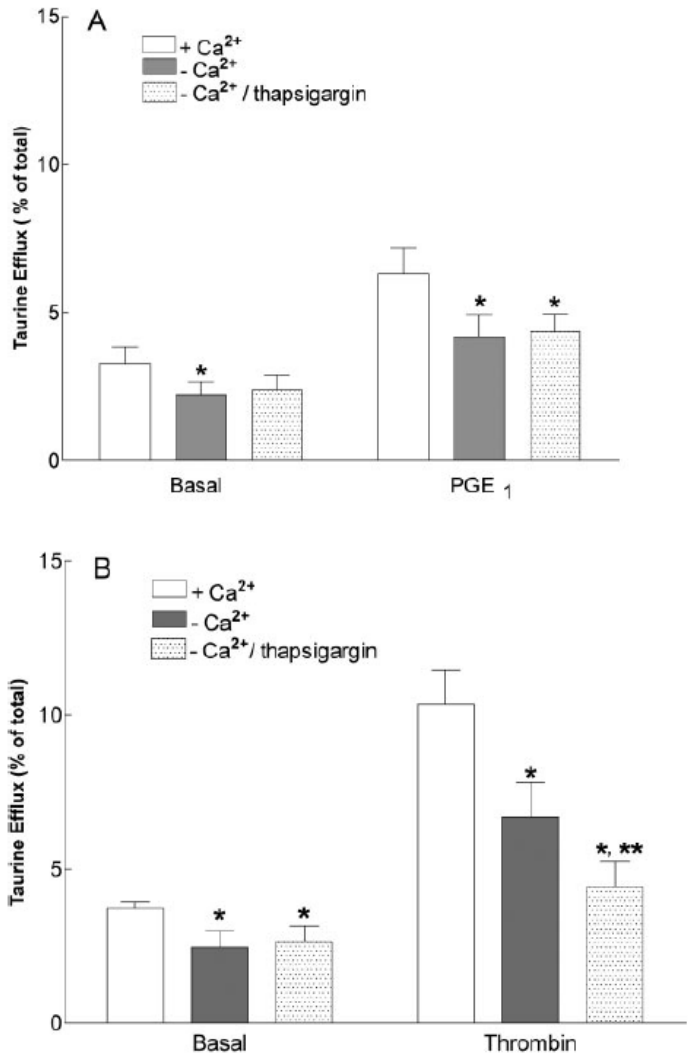

Figure A.9 The role of extracellular- and intracellular calcium in PGE_1- or thrombin-stimulated taurine efflux. Cells that had been prelabeled overnight with [^{3}H]taurine were washed in isotonic buffer A and then incubated for 5 min in hypotonic buffer A (250 mOsM) in the presence (open bars) or absence (filled bars) of extracellular Ca^{2+} (Ca^{2+} was omitted from the buffer and 50 µM EGTA added) prior to the addition of either (A) PGE_1 (20 µM) or (B) thrombin (1.25 nM), as indicated. In some experiments, cells were preincubated for 5 min in Ca^{2+}-free hypotonic buffer A (Ca^{2+} omitted and 50 µM EGTA added) in the presence (stippled bars) of 1 µM thapsigargin (TG) prior to the addition of agonist. Incubations were then allowed to proceed for an additional 10 min, reactions terminated and results expressed as taurine efflux (percentage of total soluble radioactivity). Values shown are the means ± S.E.M. for 10 independent experiments for PGE_1 or 6 independent experiments for thrombin, each performed in triplicate**,*** different from Ca^{2+}-containing control incubations, $p<0.05$ (by one-way ANOVA followed by Dunnett's multiple comparison test); **, different from Ca^{2+}-free incubations, $p<0.01$ (by one-way ANOVA followed by Dunnett's multiple comparison test).

the presence and absence of thapsigargin, respectively, $p < 0.01$, Fig. A.9).

To examine the involvement of PKC in PGE_1- and thrombin-stimulated taurine efflux, L cells were preincubated in isotonic buffer A for 15 min with 10 μM chelerythrine prior to agonist challenge under hypotonic conditions. Chelerythrine had no inhibitory effect on basal, PGE_1- or thrombin-stimulated taurine efflux and preincubation of L cells with the PKC inhibitor slightly enhanced all three parameters (Fig. A.10). When calculated relative to their respective controls, the addition of PGE_1 increased taurine efflux to 306 ± 36 and 320 ± 44% of control in the absence or presence of chelerythrine, respectively, whereas the corresponding values for thrombin-stimulated taurine efflux were 428 ± 125 and 430 ± 136% of control, respectively, n=5, Fig. A.10. In addition, chelerythrine had no effect on mAChR-stimulated taurine release from L cells that had been stably transfected with the m_3 mAChR (data not shown). The ability of Gö 6983, a highly potent cell-permeant PKC inhibitor, to inhibit PGE_1-stimulated taurine efflux was also examined. When cells were pretreated for 15 min with 1 μM Gö 6983 and then challenged with PGE_1, agonist-stimulated release of taurine was not significantly reduced (278 ± 12 and 284 ± 27% of basal in the absence and presence of Gö 6983, respectively, n=6). Preincubation of L cells with 1 μM bisindolylmaleimide, another broad spectrum PKC inhibitor, also had no inhibitory effect on basal, PGE_1- or thrombin-stimulated taurine efflux (data not shown).

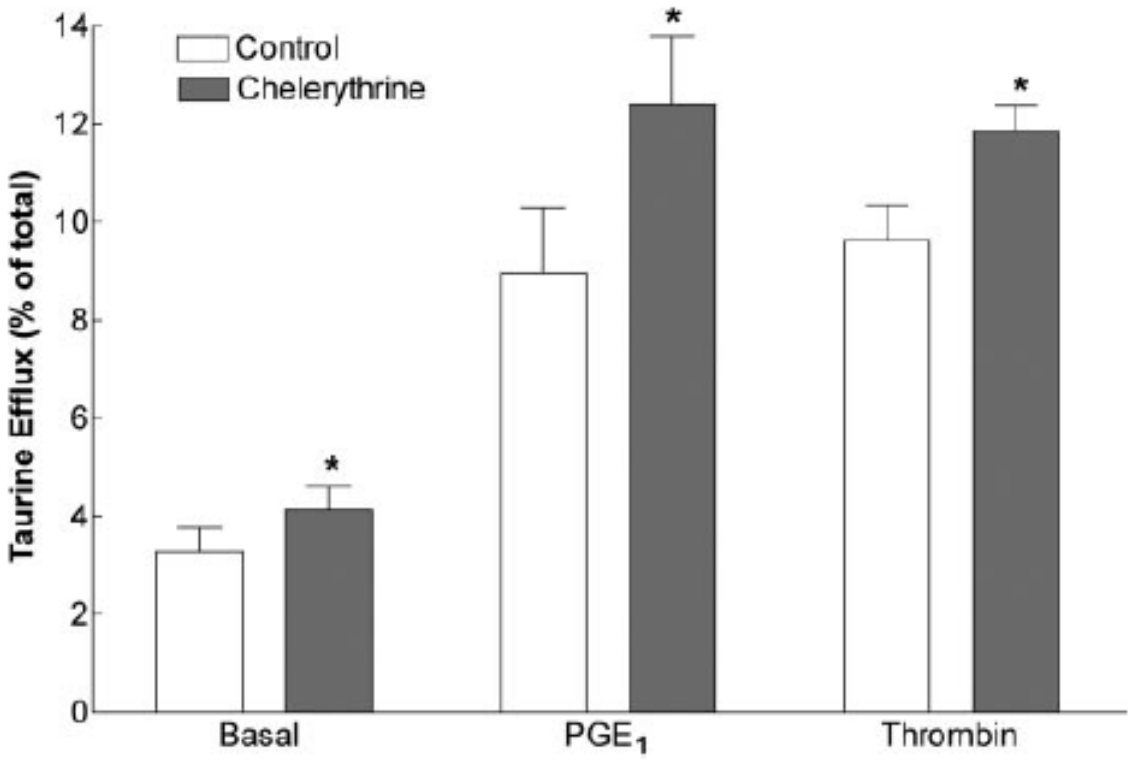

Figure A.10 Inhibition of PKC does not attenuate either PGE_1-or thrombin-stimulated taurine efflux. Cells were pretreated in the absence or presence of 10 µM chelerythrine in isotonic buffer A for 15 min before incubation of cells in hypotonic buffer A (250 mOsM) in the absence (control: open bars) or presence of chelerythrine (filled bars) in the presence or absence of PGE_1 or thrombin, as indicated. Reactions were terminated after 10 min and taurine efflux monitored. Results are expressed as taurine efflux (percentage of total soluble radioactivity) and are the means ± S.E.M for five independent experiments, each performed in triplicate. *, different from control release, $p<0.01$ (by paired Student's *t* test).

Discussion

Although the role of organic osmolyte release in volume regulation following hypoosmotic stress has been extensively studied, only recently has the possibility that this process is subject to neurohumoral control been systematically examined. To date, of the several GPCRs that have been demonstrated to facilitate the volume-dependent release of osmolytes from cells, all appear to share a common characteristic in that receptor activation triggers a mobilization of intracellular Ca^{2+}, although the precise role of Ca^{2+} in osmolyte release remains to be determined. Furthermore, maximum receptor-stimulated osmolyte release also appears to require PKC activity (Mongin and Kimelberg, 2002, 2005; Falktoft and Lambert, 2004; Heacock et al., 2004, 2006; Cheema et al., 2005). In the present study, we demonstrate that a GPCR that couples to adenylyl cyclase activation is also able to facilitate osmolyte release via a mechanism that is distinct from that previously described. Evidence to support the conclusion that prostanoid receptors present on mouse fibroblasts regulate osmolyte efflux via a cAMP-dependent mechanism is based upon three series of experimental observations. First, the ability of PGE_1 to enhance taurine efflux could be mimicked by the addition of agents that elevate intracellular cAMP concentrations via either the inhibition of cAMP breakdown or direct activation of adenylyl cyclase or, alternatively, by acting directly as cell-permeant analogs of cAMP (Fig. A.6). Moreover, when these agents were included in incubations that contained PGE_1, no further increase in osmolyte release was observed, a result consistent with a common mechanism of action. Second, of the

PGE analogs tested, only butaprost, an analog that selectively activates the prostanoid EP_2 receptor subtype that couples to activation of adenylyl cyclase, could facilitate osmolyte release. Third, the ability of either PGE_1 or 8-CPT-cAMP (but not that of either thrombin or sphingosine 1-phosphate) to stimulate taurine efflux was significantly attenuated in RAB-10 cells, which exhibit a lower activity of PKA than L cells. Since PKA is the major downstream cellular target for cAMP action, this result is a further indication that the cyclic nucleotide is the mediator of osmosensitive increases in taurine efflux. The conclusion that prostanoid receptor-mediated changes in cAMP can regulate taurine release is consistent with a previous study in which isoproterenol, a β-adrenergic agonist, was observed to increase osmolyte efflux from glial cells (Moran et al., 2001). Taken collectively, the results indicate that receptor-mediated increases in cAMP are potentially linked to the process of osmoregulation in cells.

One complication in the interpretation of our results is that, in addition to its previously documented ability to increase the concentration of intracellular cAMP in L cells (Maganiello and Vaughn, 1972; Uhler and Abou-Chebl, 1992), PGE_1 was also observed to elicit an increase in $[Ca^{2+}]_i$ (Fig. A.8). However, two lines of evidence suggest that the rise in $[Ca^{2+}]_i$ and increases in cAMP concentration are distinct events in L cells. First, the addition of forskolin, 8-CPT-cAMP or the EP_2-selective agonist, butaprost, (all of which elicit robust increases in taurine efflux) failed to mimic the ability of PGE_1 to increase $[Ca^{2+}]_i$. Second, the PGE_1–mediated increase in osmolyte release was essentially independent of both extra- and intracellular Ca^{2+} (when calculated on a fold-stimulation basis) even though the

agonist-mediated increase in Ca^{2+} was either substantially inhibited or abolished under these conditions (see Fig. A.8). In contrast, although taurine release stimulated by thrombin addition was also independent of extracellular Ca^{2+}, depletion of intracellular Ca^{2+} with thapsigargin strongly attenuated the response, a result consistent with our previous findings in astrocytoma cells (Cheema et al., 2005). The most parsimonious interpretation of these results is that L cells possess two populations of prostanoid receptors, one that couples to the activation of adenylyl cyclase, PKA activation and osmolyte release whereas a second group of receptors is linked to an increase in Ca^{2+} mobilization. It appears that the latter population of receptors does not make a significant contribution to osmolyte release in L cells. In this context, it should be noted that distinct differences in the susceptibility of GPCR-stimulated osmolyte release to depletion of intracellular Ca2+ have been observed. Thus whereas taurine release elicited by the addition of either lysophosphatidic acid or sphingosine 1-phosphate is reduced by 30-40%, the responses to ATP and muscarinic agonists are essentially abolished (Mongin and Kimelberg, 2005; Heacock et al., 2006).

PGE_1 stimulation of taurine release also appears to be independent of PKC, as determined from the inability of chelerythrine, Gö 6983 or bisindolylmaleimide to significantly inhibit either basal- or PGE_1-induced osmolyte release. The observation that inhibition of PKC also did not attenuate either thrombin- or mAChR-stimulated taurine release from L cells was unexpected and at variance with previous studies in which PKC activity was found to be necessary for the maximum release of osmolytes in response to either of these receptors (Cheema et al., 2005; Heacock et

al., 2006). One interpretation of the present findings is that PKC activity may not invariably be a pre-requisite for agonist-stimulated osmolyte release, even for Ca^{2+}-mobilizing receptors.

Although taurine release elicited by prostanoid receptor stimulation appears to differ from that exhibited by previously studied receptors in terms of its apparent lack of Ca^{2+} and PKC dependence, two features common to all receptors can be identified. The first is that, similar to osmolyte release induced by Ca^{2+}-mobilizing agonists, a VSOAC appears to mediate osmolyte efflux as indicated by the ability of non-selective anion channel inhibitors, such as DDF and NPPB, to block PGE_1-stimulated taurine efflux. This conclusion is strengthened by the observation that DCPIB, a highly selective inhibitor of VSOAC (Decher et al., 2001) also significantly inhibits PGE_1-stimulated taurine efflux (Fig. A.5). A second characteristic shared by both the prostanoid receptor and those receptors primarily linked to Ca^{2+}mobilization is a reduction in the osmotic threshold for osmolyte release following receptor activation. Thus, in the absence of PGE_1 addition, the ability of L cells to significantly respond to hypoosmotic stress is restricted to a relatively large reduction in osmolarity (>33%) whereas in the presence of the agonist, osmolyte release occurs when the osmolarity is reduced by <15% (Fig. A.4). The present results are consistent with data previously obtained for osmolyte release following agonist activation of other GPCRs such as the P2Y purinergic, m3 mAChR, PAR-1, sphingosine 1-phosphate and lysophosphatidic acid receptors (Mongin and Kimelberg, 2002, 2005; Heacock et al., 2004, 2006; Cheema et al., 2005) and also that elicited following activation of the epidermal growth factor receptor (Franco et

al., 2004). These results indicate that, regardless of the underlying mechanism(s) of activation, receptors coupled to osmolyte efflux share a common property in their ability to facilitate osmolyte release from cells in response to relatively small changes in osmolarity, such as those that are likely to be encountered *in vivo*.

In summary, the results in the present study provide evidence that prostanoid receptors coupled to an increase in cAMP concentration can facilitate osmolyte release from L cell fibroblasts in a volume-dependent manner by a mechanism that is distinct from that previously described for Ca^{2+}-mobilizing receptors. These results raise the possibility that cells may utilize multiple cell signaling mechanisms to regulate their volume in the face of hypoosmotic challenge.

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