Characterization of

a novel osmotically-evoked phospholipase C pathway

and

Slack and Slick-mediated Na^+-activated K^+ currents

in rat supraoptic neurons

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By

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Abstract

The magnocellular neurosecretory cells (MNCs) of the hypothalamus are important players in systemic osmoregulation that strives to stabilize water and salt levels inside the mammalian body. In accordance with the physiological osmotic needs, the MNCs adopt changes in their electrical activity to regulate the systemic release of vasopressin (VP) and oxytocin (OT) hormones, which act on the kidneys to control urine and sodium excretion, respectively. Although MNCs are known to exhibit osmotically-evoked changes in their membrane bound TRPV1 channels and intracellular cytoskeleton proteins, which confer them intrinsic osmosensitive properties, the identity and role of osmotically-evoked changes in their second messenger systems are less clear. In the first part of this Ph.D. thesis, I present evidence for the presence of a novel osmotically-evoked and Ca^{2+}-dependent phospholipase C (PLC) signaling pathway in the rat MNCs using immunocytochemical methods. Using patch clamp methods, I have also shown that this osmotically-evoked PLC pathway acts in a feed forward manner to potentiate the intrinsic osmosensitivity of MNCs by contributing to the activation of TRPV1 channels. Although MNCs are known to adopt changes in their electrical activity in accordance with the physiological needs of VP and OT, the mechanisms regulating the transition in their electric behaviour are unclear. It is therefore important to identify all the different ion channels that are possibly present in MNCs and could potentially contribute to their electrical behave;/. In the second part of this Ph.D. thesis, using patch clamp methods I have demonstrated novel evidence for the presence of Na^+-activated K^+ (K_{Na}) channels in rat MNCs. Since K_{Na} channels contributes to activity-dependent after hyperpolarizations (AHPs) and shaping of firing behaviour in other neurons, it is possible that they could also play an important role in regulating the electrical behaviour of MNCs. In summary, this Ph.D. thesis contributes to a better understanding of mechanisms that regulates both the osmotic physiology and electric behaviour of the MNCs and thus the overall process of systemic osmoregulation in the body.

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4-AP	4-Aminopyridine
AHP	Afterhyperpolarizing potential
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
AP	Action potential
ATP	Adenosine triphosphate
BAPTA-AM	1, 2-Bis (2-aminophenoxy) ethane-N, N, N', N'-tetra acetic acid (acetoxymethyl ester)
BDNF	Brain-derived neurotrophic factor
BK	Large-conductance Ca^{2+}-activated K^+ channel
Ca^{2+}	Calcium ion
cAMP	Cyclic adenosine monophosphate
cGMP	Cyclic adenosine monophosphate
Cl^-	Chloride ion
CNS	Central nervous system
CO	Carbon monoxide
CSA	Cross-sectional area
CVOs	Circumventricular organs
DAG	Diacyl-glycerol
DAP	Depolarizing afterpotential
DHP	Dihydropyridine
DIC	Differential interference contrast
DiC_8-PIP_2	Dioctanoyl PIP_2
DRG	Dorsal root ganglion

EAG	*ether-a-go-go*-related gene
ECF	Extracellular fluid
EGTA	Ethylene glycol tetra acetic acid
EPSCs	Excitatory post synaptic currents
fAHP	Fast after hyperpolarizing potential
fDAP	Fast depolarizing afterpotential
FRET	Forster resonance energy transfer
GABA	γ-Aminobutyric acid
GIRK	G protein-coupled inwardly-rectifying potassium
GlyR	Glycine receptor
GPCR	G protein-coupled receptor
GTP	Guanosine-5'-triphosphate
GYG	Glycine-Tyrosine-Glycine sequence
HAP	Hyperpolarizing afterpotential
HPLC	High performance liquid chromatography
HVA	High voltage activated
Hz	Hertz
ICF	Intracellular fluid
IGF-1	Insulin like-growth factor 1
IK	Intermediate conductance
IP_3	Inositol 1, 4, 5-trisphosphate
K^+	Potassium ion
K_{ATP}	ATP sensitive potassium channel
K_{Ca}	Calcium activated potassium channel
K_{ir}	Inwardly-rectifying potassium channel

K_{Na}	Sodium-activated potassium channel
K_V7	Voltage-gated potassium channel belonging to family 7
LDCVs	Large dense core vesicles
Li^+	Lithium ion
LVA	Low voltage activated
mAHP	medium after hyperpolarizing potential
MNC	Magnocellular neurosecretory cell
MnPO	Median preoptic nucleus
mosmol kg^{-1}	Milliosmoles per kilogram of solvent
mosmol L^{-1}	Milliosmoles per liter of solvent
Na^+	Sodium ion
Na_X	Sodium sensitive channel
NKCC	Sodium potassium chloride cotransporter
NMDA	N-methyl-D-aspartate
NO	Nitric oxide
NP	Neurophysin
NPs	Neuropeptides
NSF	N-ethylmaleimide sensitive fusion protein
NTs	Neurotransmitters
NTS	Neurotensin
OKC:	Osmosensitive potassium current
OT:	Oxytocin
OVLT	Organum vasculosum of the lamina terminalis
PACAP	Pituitary adenylate cyclase-activating polypeptide
PBS	Phosphate-buffered saline

PCR:	Polymerase chain reaction
PGC	PIPES-glucose-calcium buffer
PH	Pleckstrin homology
pH	power of hydrogen
PI4P	Phosphatidylinositol 4-phosphate
PIP_2	Phosphatidylinositol 4, 5-bisphosphate
PIPES	Piperazine-N, N'-bis (2-ethanesulfonic acid)
PKC	Protein kinase C
PLC	Phospholipase C
PMA	Phorbol-12-myristate-13-acetate
POMC	Pro-opiomelanocortin
PVN	Paraventricular nucleus
RR	Ruthenium red
RTKs	Receptor tyrosine kinases
RVD	Regulated volume decrease
RVI	Regulated volume increase
sAHP	Slow after hyperpolarizing potential
sDAP	Slow depolarizing afterpotential
SEM	Standard error of mean
SFO	Subfornical organ
SIC	Stretch-inactivated cation channel
SK	Small conductance
SNARE	Soluble NSF attachment protein receptor
SON	Supraoptic nucleus
SVs	Synaptic vesicles

TASK	TWIK-related acid-sensing potassium channel
TEA:	tetraethyl ammonium
TRAAK	TWIK-related arachidonic acid activated potassium
TREK	TWIK-related potassium channel
TRP	Transient receptor potential channel
TRPA	Transient receptor potential channel having Ankyrin repeats
TRPC	Transient receptor potential channel canonical
TRPM	Transient receptor potential channel melastatin
TRPV	Transient receptor potential channel vanilloid
TTX	Tetrodotoxin
VDCCs	Voltage dependent calcium channels
VP	Vasopressin

CHAPTER 1

GENERAL INTRODUCTION

1.1 Body water distribution and the need for water balance

Water is the most abundant compound on earth and is among the most vital constituents that have facilitated evolution and sustenance of life on our planet (Selvaratnam, 1998; Rautela, 2000; Ruse *et al.*, 2009). The importance of water in our lives can be readily appreciated by the fact that water alone constitutes about 60% of the average human body weight. The body water performs a number of functions: it acts as a solvent in our blood for transportation of nutrients and gases to the cells; it acts as a medium for excreting the metabolic wastes from our body in the form of urine and faeces; its large heat capacity allows us to efficiently regulate changes in our body temperature in both warm or cold environments; it acts a lubricant and shock absorbent for the joints thereby enabling us to walk and move smoothly; it provides an appropriate aqueous medium for the biochemical reactions that are critical for our survival (Kang, 2012; Sizer *et al.*, 2012; Herlihy, 2014).

The body water is present either in the intracellular fluid (ICF) or the extracellular fluid (ECF) compartments. While the ICF represents all body fluids that are present inside the cells (cytoplasmic fluids), the ECF represents all body fluids that are present outside the cells. The ECF has been further subdivided into two fluid compartments – the vascular fluid (the blood plasma present within the blood vessels) and the interstitial fluid (the fluid that bathes and surround the cells in various tissues and is present outside the vascular system). A third minor ECF compartment is the transcellular fluid compartment, which includes specialized fluids like sweat, synovial fluid and urine that are separated from the blood plasma by an additional epithelial layer unlike the interstitial fluids, which are separated from the blood plasma by only the endothelial

cell layer of the blood vessels (Khurana, 2006; Rhoades *et al.*, 2012).

Although both the ECF and ICF in our body are largely made up of water, they also contain numerous other constituents like various ions, nutrients, gases, and proteins, which are critical for performing specific cellular functions and thereby keeping us alive (Preston *et al.*, 2013). Moreover the ECF and the ICF have different physiological compositions and preventing changes in them is essential for our survival. For example, the ECF has high relative Na^+ and Cl^- concentrations while the ICF has high relative K^+ concentration. Maintenance of these Na^+ and K^+ concentration gradients is extremely important for proper generation and conduction of action potentials (APs) in the nervous system (Ignatavicius *et al.*, 2002). Similarly ECF has a relatively higher Ca^{2+} concentration and since the strength of heart contractions is directly dependent on the concentration gradient of Ca^{2+} (Khurana, 2014a), changes in the ECF composition can have significant impacts on the cardiovascular system. One of the ways the body preserves the integrity of the ECF and the ICF is by keeping them separated by cell membranes that act as physical barrier and have a highly selective permeability (Sherwood *et al.*, 2010). The selective permeability of cell membranes ensures that impermeable cellular contents like these ions don't mix and cause changes in the composition of these fluids, while at the same time permeable materials like respiratory gases, metabolic wastes and water can freely move across them to perform the necessary physiological functions whenever needed.

Although the individual composition of the ECF and the ICF are quite different, the body strives to maintain similar osmotic strength in these fluids. The osmotic strength of a solution is commonly reported in terms of osmolality, which is defined as concentration of total solutes that are present per kilogram weight of a solution (Bourque, 2008) and/or tonicity, which is defined as the concentration of the membrane- impermeant solutes that are present per kilogram weight of

a solution (Lerma *et al.*, 2012). Both osmolality and tonicity are expressed in osmol kg^{-1} (osmoles per kilogram of solution) units, which indicates presence of defined number of moles of a solute per kilogram weight of the solution. Although both osmolality and tonicity are different concepts, the terms are often used interchangeably in the context of the biological fluids as most of the solutes present in these fluids are membrane-impermeant. A solution having a relatively higher concentration of the impermeant solutes is said to be hypertonic, while one having a relatively lower concentration of them is referred to as a hypotonic solution. When such solutions having different osmotic strengths are separated by a semipermeable barrier like biological cell membranes (which are more permeable to water molecules than ions at rest), there is a tendency for water to move from the hypotonic side to the hypertonic side through a process termed as osmosis (Kent, 2000). Osmosis is facilitated by a family of membrane proteins called aquaporins, which act as water channels for mediating the water flow (Alberts *et al.*, 2014). Osmosis continues till there is an equilibration of solute concentration on both sides of the membrane. Understandably, if the two solutions separated across the membrane are isotonic to begin with (i.e. they have a similar concentration of impermeant solutes), there is no osmosis and thus changes in their composition due to water movement is spared. The matching of the osmotic strength between ECF and ICF is therefore a physiological mandate for preserving the difference in their compositions, which otherwise can be altered by osmotic water movement.

However this does not mean that the ECF osmolarity is rigid and cannot change; it is variable and can rapidly change depending on our daily activities. For example, when we drink too much fluid the ECF osmolarity transiently decreases and when we eat a salt-rich diet or undergo evaporative water loss the ECF osmolarity would increase causing osmotic imbalance in the body (Prager-Khoutorsky *et al.*, 2015). Although some of this osmotic imbalance is rapidly restored by

the unopposed water movement that occurs due to generation of an osmotic gradient between ECF and ICF, it is not a sufficient physiological solution to this problem. This is because free water movement from or into cells can itself lead to unwanted cellular shrinkage or swelling, which can grossly impair biological functions by causing forced structural changes in the intracellular proteins and organelles and sometimes even triggering programmed cell death (Hanninen *et al.*, 2009). Since perturbations in the ECF osmolarity can lead to unwanted behavioral changes, organ damage and much more serious health issues for the individual (Bourque, 2008), there needs to be mechanisms in place that can help restore the ECF osmolarity to normal values without causing volumetric changes in the cells.

To prevent volumetric changes during osmotic insults, many body cells exhibit regulatory volume increase (RVI) and regulatory volume decrease (RVD) mechanisms, which tend to oppose the osmotic shrinkage and swelling, respectively. These acute volume regulatory mechanisms are caused by fluxes of ions/osmolytes through volume-sensitive channels and transporters in their cell membrane (Lang, 2007; Hanninen *et al.*, 2009). Although these volume regulatory mechanisms appear useful from a volumetric point of view, their activation also risks alteration in the ionic gradients and changes in overall composition of both ECF and ICF, which is undesirable as discussed before.

1.1.1 Systemic osmoregulation:

To circumvent the volumetric issues during osmotic changes in the body, all mammals have therefore evolved an effective, centralized osmoregulatory process wherein specialized osmotic centres are enabled to detect changes in ECF osmolarity and then accordingly initiate corrective changes in the body to restore ECF osmolarity without having the body cells undergo the unwanted volumetric changes. The homoeostatic process that maintains this stable osmotic

environment inside the body is referred to as systemic osmoregulation and is a key survival process (Bourque, 2008).

All mammals strive to maintain a constant ECF osmolality to prevent damage to their cellular structures and functions. The ECF osmolality is usually maintained around a species-specific physiological set point (e.g. 280 mosmol kg^{-1} in humans, 295 mosmol kg^{-1} in rats, 310 mosmol kg^{-1} in mice) by the homoeostatic process of systemic osmoregulation (Bourque *et al.*, 2007; Bourque, 2008). Since water and ions are the prime determinants of osmotic strength, systemic osmoregulation operates by regulating various processes that affect our daily intake and excretion of water and salts. This is achieved by execution of a dual control over the passive physiological (urination and sweat) and active behavioural (drinking fluids, salt preference and aversion) processes that work in concert to bring the ECF osmolality back to normal (Voisin *et al.*, 2002). For example, drinking too much fluid causes a decrease in the osmotic strength of the ECF that invokes an increase in urination to decrease the water load in body while eating salty food increases the ECF osmolality, which can invoke an increase in thirst behaviour and decrease in urination to increase the water content in the body. The homeostatic working of systemic osmoregulation is summarized in Figure 1.1.

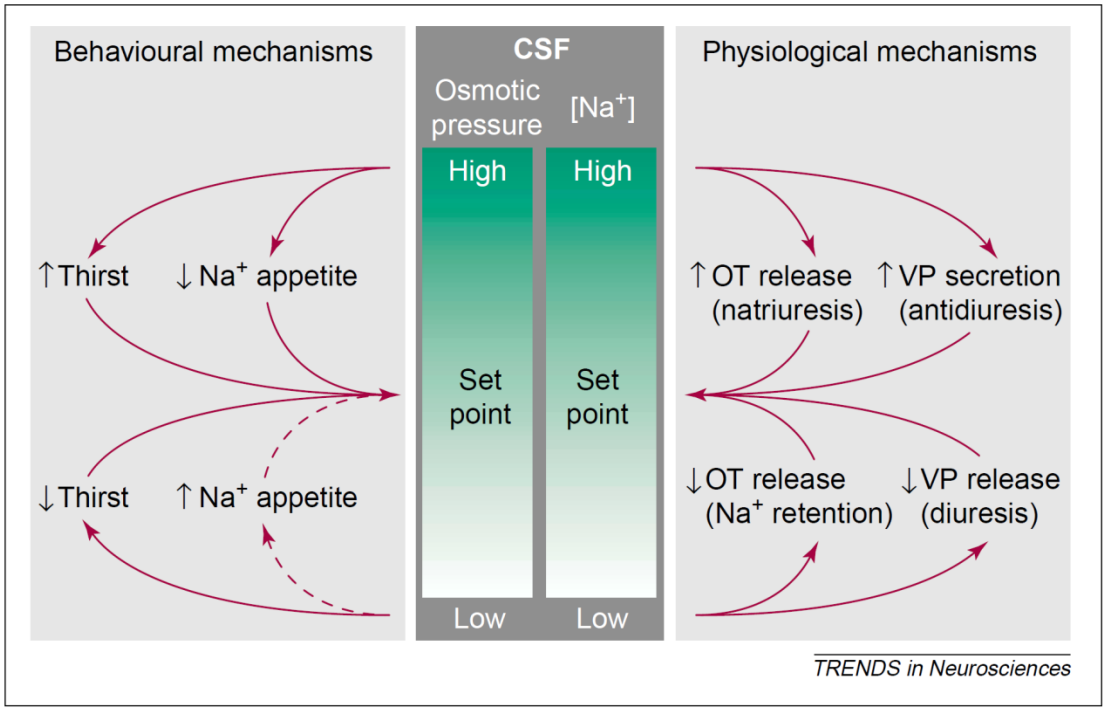

Figure 1.1. Systemic osmoregulation restores osmotic balance by regulating behavioural and physiological mechanisms in the body.
The salt and water levels in the mammals are usually maintained at a species-specific physiological set point. Specialized sensory mechanisms detect the difference between pre-established physiological set-point and prevailing values of CSF [Na^+] and/or osmolality. Deviations from the physiological set-point activate proportionate behavioural and physiological responses to achieve homeostasis. The dashed line indicates that experimental evidence for this response is presently incomplete. Behavioural responses involve changes in water intake and/or Na^+ intake; physiological responses involve changes to the rate of water loss ((controlled by vasopressin (VP)) and/or Na^+ excretion ((controlled in part by oxytocin (OT)). Adapted with permission from (Voisin *et al.*, 2002).

Although many different brain and peripheral centers contribute to systemic osmoregulation (Bourque, 2008), the MNCs (magnocellular neurosecretory cells) located in SON (supraoptic nucleus) and PVN (paraventricular nucleus) of the hypothalamus are generally accepted to be key players in this process and are discussed below.

1.2 MNCs and their hormones VP and OT

The MNCs are key effectors of osmoregulation because depending on changes in the ECF osmolality, they regulate the systemic release of two principal osmoregulatory hormones named VP (vasopressin) and OT (oxytocin) in the body (Bourque *et al.*, 1994). The systemic levels of VP and OT are high when ECF osmolality increases and their levels drop when the ECF osmolality decreases (Bourque *et al.*, 1994; Bourque *et al.*, 1997). VP and OT are synthesized in MNC somata and released from their axon terminals in the posterior pituitary following electric activity in their somata (Dreifuss *et al.*, 1971; Nordmann, 1977; Gross *et al.*, 1986).

VP and OT are released into the systemic circulation and travel through the vascular system to their specific receptor targets in the kidney to regulate water excretion (Nielsen *et al.*, 1995) and natriuresis (Verbalis *et al.*, 1991), respectively. VP, also called ADH (antidiuretic hormone) increases the permeability of the collecting ducts to water, reducing the renal excretion of water and thus promoting water conservation. Specifically, VP acts on the VP receptors (V2 type) that are present in the basolateral membrane of cells in the cortical and medullary collecting tubules. This receptor agonist action activates adenyl cyclase and cAMP (cyclic adenosine monophosphate - a second messenger molecule) is formed. This causes translocation and fusion of specific vesicles present in the cytoplasm of these cells into their apical membranes. Since the vesicles contain water channels (aquaporins), their insertion makes the apical membrane permeable to water and therefore excess water present in the lumen can

be reabsorbed into the cells following the osmotic gradient (Kuwahara *et al.*, 1995; Noda *et al.*, 2006; Park *et al.*, 2015). Conversely, when the ECF osmolarity has decreased and the body needs to remove excess water, MNCs decrease VP secretion, which causes removal of water channels from the apical membrane to reduce water reabsorption. This dynamic shuttling of water channels in the kidney cells, in response to plasma VP levels, is primarily responsible for the passive osmoregulatory control in the body. Although the VP-dependent translocation of aquaporins is a rapid event, VP has also been shown to regulate the expression of aquaporin proteins during more prolonged osmotic changes in the body (Promeneur *et al.*, 2000; Hasler *et al.*, 2002). On the other hand, OT, although primarily known as a maternal hormone and considered important for regulating milk release and uterine contractions during lactation and parturition (Brown *et al.*, 2013), also invokes antidiuretic actions by directly stimulating vasopressin receptors to increase apical trafficking of aquaporins (Li *et al.*, 2008) perhaps due to its structural similarity with VP (Sasaki, 2008). Moreover, in rats and dogs, OT can also stimulate sodium excretion by acting on various ion transport mechanisms in the nephrons (natriuresis) and thus contribute to lowering the ECF osmolarity (Kleinman *et al.*, 1980; Verbalis *et al.*, 1991; Bourque *et al.*, 1997).

MNCs are called magnocellular due to their large cell size (diameters in the range of 15–40 µm). A previous study has shown that >97% of all cells that were isolated from the SON and have a cross sectional area (CSA) larger than 160 μm^2 are MNCs (Oliet *et al.*, 1992). It has been shown that there are a total of about 6000-7000 MNCs in rats (Rhodes *et al.*, 1981) and about 80000-100,000 in humans (Manaye *et al.*, 2005). The majority of the SON MNCs are VP secreting while majority of the PVN MNCs are OT secreting (Laycock, 2010). Furthermore, within the SON the VP MNCs are thought to be clustered in ventral and caudal parts while the OT MNCs are

predominantly present in the rostro-dorsal parts of the nucleus (Ghamari-Langroudi *et al.*, 2004). Although VP and OT are secreted by distinct MNC subpopulations, there aren't any morphological differences between the two cell types. There also have been reports suggesting that about 1-3% of MNCs in these nuclei could secrete both VP and OT and that this number can increase up to 15% under physiologically-demanding conditions (Kiyama *et al.*, 1990; Mezey *et al.*, 1991; Gainer, 1998; Glasgow *et al.*, 1999). The physiological significance of co-expression of VP and OT in some of these MNCs is unclear at present and awaits further studies (Castro, 2001). Although MNCs are the predominant neuronal population in SON, there are another group of neurons called parvocellular neurons that are present along with the MNCs in the PVN. The parvocellular neurons usually have smaller cell bodies (10-15 μm diameter) and are known to project to the median eminence where they release regulatory hormones that regulate the systemic levels of thyrotropin, corticotrophin, prolactin and luteinizing hormones (Burbach *et al.*, 2001).

1.3 MNC electrical activity and mechanisms regulating their electrical activity

The axonal projections of MNCs releases VP and OT in the posterior pituitary following electrical activity in the MNCs soma (Dreifuss *et al.*, 1971) through exocytosis of large dense-core vesicles that occurs in a Ca^{2+} dependent exocytotic fashion (Hsu *et al.*, 1996). Since the MNC terminals do not sustain intrinsic repetitive AP discharge (Bourque, 1990), the secretion of VP and OT are primarily determined by the frequency and pattern of AP discharge initiated in the MNC somata (Bicknell, 1988). The MNCs exhibit a variety of firing patterns, an integrated output of which determines the circulating levels of both VP and OT (Brown *et al.*, 2013). The different firing patterns exhibited by the MNCs are summarized in Figure 1.2.

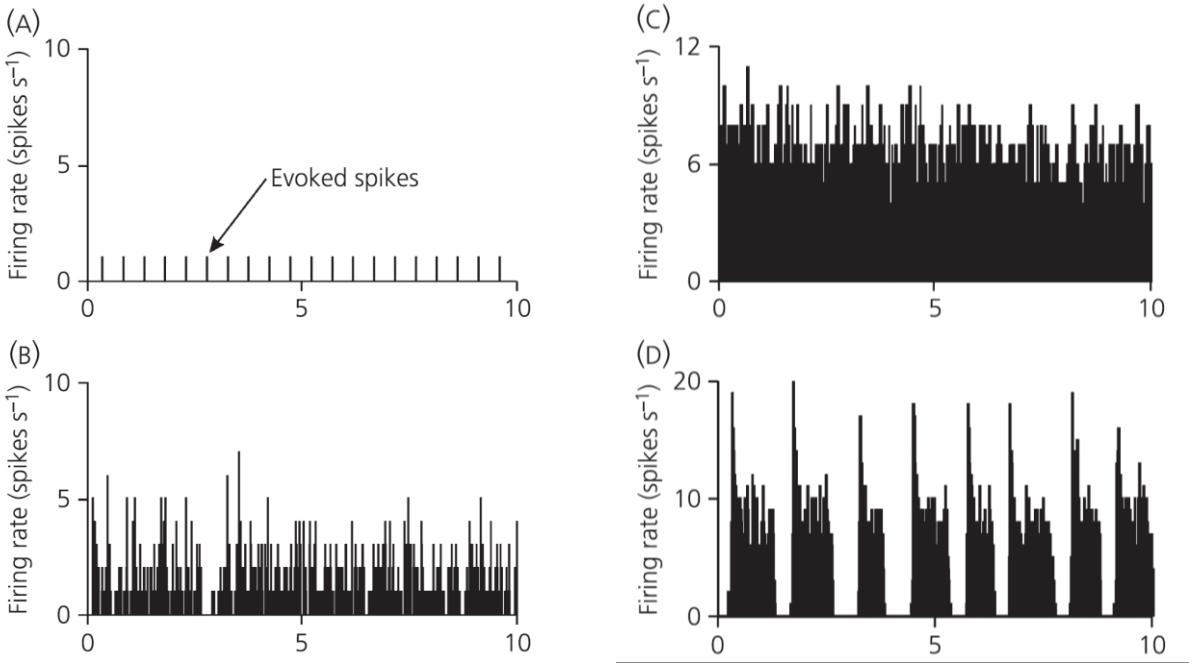

Figure 1.2. MNCs exhibit a variety of firing patterns.
The ratemeter records show individual examples of the spontaneous electrical activity (averaged in 1-s bins) of MNCs obtained in urethane-anaesthetized rats. Under basal conditions, MNCs exhibit a range of activity patterns from silent (A), irregular (varying firing frequency) (B) continuous (relatively constant frequency) and (C) phasic bursts (alternate periods of activity and silence). Adapted with permission from (Brown *et al.*, 2013).

Although MNCs under basal conditions display all four types of firing patterns shown in Figure 1.2, they have a tendency to adopt specific firing patterns depending on the physiological needs. For example, both VP and OT MNCs fire irregularly and infrequently when plasma osmolality is near the normal physiological set point of 295 mosmol kg^{-1} in rats, and both adopt higher frequency firing patterns as the external osmolality increases (Poulain *et al.*, 1982; Bourque *et al.*, 1997) leading to increased VP and OT levels in the systemic circulation (Verbalis *et al.*, 1986). Although the increase in firing frequency of both VP and OT MNCs enhances the systemic levels of their respective neuropeptides, their release is maximized by burst firing patterns (Bicknell, 1988), which both types tend to adopt, albeit under different physiological conditions. For example, most VP MNCs have a tendency to adopt a phasic burst pattern during osmotically stimulating conditions while most OT MNCs tend to adopt a high frequency burst pattern during partition and lactation to promote uterine contractions and milk release in females, which is the major physiological function of OT in the body (Brimble *et al.*, 1977; Wakerley *et al.*, 1978; Belin *et al.*, 1984; Jiang *et al.*, 1995; Russell *et al.*, 1998). The burst pattern in MNCs is characterized by periods of intense activity that are followed by silent periods of approximately equal duration (Brown, 2004; Brown *et al.*, 2006). Although bursts in OT MNCs have higher firing frequency and are usually shorter than the bursts in VP MNCs, burst patterns in both VP and OT MNCs are believed to maximize both their secretion and prevent secretory fatigue by intermittently allowing the MNCs to retune their secretory machinery during silent periods (Brown *et al.*, 2013). Although many activity-dependent ionic currents and autocrine mechanisms have been proposed to contribute to phasic firing patterns of MNCs, the bases of their bursting behaviours are still incompletely understood (Brown *et al.*, 2013).

1.3.1 Activity-dependent ionic mechanisms

The MNCs exhibit an activity-dependent current termed the depolarizing after potential (DAP), which is thought to provide a continuous excitatory trigger to the MNCs during their high frequency firing (Armstrong *et al.*, 1994; Li *et al.*, 1995; Li *et al.*, 1997b; Li *et al.*, 1997a; Ghamari-Langroudi *et al.*, 1998; Ghamari-Langroudi *et al.*, 2002; Teruyama *et al.*, 2007). Besides the excitatory DAP, other activity-dependent hyperpolarizing currents, commonly grouped as after hyperpolarizing potentials (AHPs), have also been reported in MNCs and are thought to a provide an inhibitory control during high frequency firing of MNCs (Kirkpatrick *et al.*, 1996; Dopico *et al.*, 1999; Ghamari-Langroudi *et al.*, 2004; Greffrath *et al.*, 2004; Ohbuchi *et al.*, 2010). MNC firing patterns, especially the burst firing are considered to be dependent on a concerted interplay of both DAPs and AHPs that are apparently activated in the MNCs in an activity-dependent and Ca^{2+}-dependent fashion (Brown *et al.*, 2013). The properties and physiological mechanisms of the DAP and the AHPs are detailed below.

1.3.1.1 Depolarizing after potentials (DAPs)

The DAP that develops after individual AP spikes in MNCs was first reported in 1983 by Andrew *et al.* It has been proposed that these individual DAPs after continuous AP spikes can summate to cause a transient elevation of the membrane potential for tens of seconds to form a plateau potential, which provides the constant excitatory trigger that allows the generation of burst firing in MNCs (Andrew *et al.*, 1983; Armstrong *et al.*, 2010). The generation of the DAP and its contribution to burst generation are summarized in Figure 1.3.

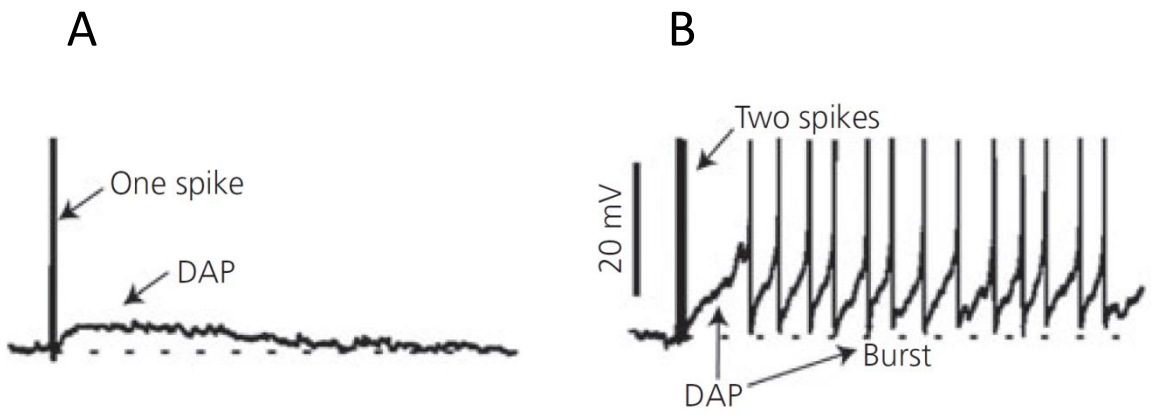

Figure 1.3. Depolarizing after potential (DAP) and its contribution to burst generation.
(A) After each individual spike in most MNCs there is a prominent postspike depolarizing after potential (DAP) that develops relatively slowly over a few seconds. (B) The individual DAPs after multiple spikes can summate to generate a depolarizing plateau potential (not shown) that could contribute to the sustained excitation of MNCs during burst firing. Adapted with permission from (Armstrong *et al.*, 2010).

Since the DAP was found to be independent of axonal conduction or chemical synapses, it was thought to be an intrinsically-activated mechanism (Andrew *et al.*, 1983). Although both VP MNCs and OT MNCs have been demonstrated to exhibit DAPs in rat hypothalamic slices and acutely isolated MNCs they are reportedly more common in VP MNCs (Oliet *et al.*, 1992; Erickson *et al.*, 1993; Armstrong *et al.*, 1994). VP MNCs have further been shown to have two components within the DAP that vary in their activation kinetics and can be pharmacologically identified (Armstrong *et al.*, 2010). For example, VP MNCs have been shown to have a caesium-insensitive large amplitude fast DAP (fDAP) that lasts for a few hundred milliseconds and a caesium-sensitive smaller amplitude slow DAP (sDAP) that lasts for several seconds (Teruyama *et al.*, 2007). Although summation of the sDAP is thought to contribute to the phasic firing patterns in MNCs, the physiological significance of the fDAP component in VP MNCs is unclear at present (Armstrong *et al.*, 2010).

The DAP is a Ca^{2+}-dependent current that has been shown to depend on Ca^{2+} influx through voltage-dependent calcium channels (VDCCs) and also on Ca^{2+} release from intracellular stores (Bourque, 1986; Andrew, 1987; Li *et al.*, 1995; Li *et al.*, 1997b; Li *et al.*, 1997a; Li *et al.*, 1999). Since the amplitude and duration of the DAP were affected by the numbers of APs and the membrane potential preceding the DAP (Andrew, 1987; Li *et al.*, 1997b; Li *et al.*, 1997a), it is thought be an activity-dependent current. The two likely candidates that have been proposed for mediating the fDAP are TRPM4 and TRPM5 channels that are known to conduct an outwardly rectifying non-specific cationic current (Teruyama *et al.*, 2007; Armstrong *et al.*, 2010). Moreover despite the fact that Ca^{2+} seems to be important for generation of both types of DAPs in MNCs, there have been conflicting reports on whether the sDAP is generated due to activation of Ca^{2+}-dependent depolarizing currents or due to inhibition of Ca^{2+}-dependent hyperpolarizing currents

that follows the individual spikes (Andrew, 1987; Li *et al.*, 1997b; Armstrong *et al.*, 2010). In summary, although the DAP seems to be important for phasic firing in the MNCs, its identity and ionic mechanisms are unclear and could involve multiple pathways.

1.3.1.2 After hyperpolarization potentials (AHPs)

Although DAPs could explain the increase in excitability that is needed for initiation of rapid burst firing, it is clear that additional inhibitory mechanisms should be present that could progressively decrease the effect of DAPs and help make the MNCs refractory (silent) at specific intervals for their adoption to burst firings and also facilitate the spike frequency adaptation that is characteristic of burst firing (Kirkpatrick *et al.*, 1996; Brown, 2004). The specific mechanisms regulating the bursting frequency and duration are however not completely understood and could involve a number of activity-dependent AHPs as explained below (Roper *et al.*, 2004; Armstrong *et al.*, 2010) and somatodendritic release of dynorphin, which I will explain in the following sections.

MNCs have been shown to exhibit at least three different types of AHPs - a large amplitude fast AHP (fAHP) that lasts < 10 ms and usually follows a single AP spike (Greffrath *et al.*, 2004), a small-amplitude medium AHP (mAHP) that lasts several hundred ms and an even smaller amplitude slow AHP (sAHP) that last few seconds, both of which are commonly seen after multiple AP spikes (Kirkpatrick *et al.*, 1996; Greffrath *et al.*, 1998; Ghamari-Langroudi *et al.*, 2004). The generation of all three AHPs are summarized in Figure 1.4 and their activation mechanism are explained in the following sections.

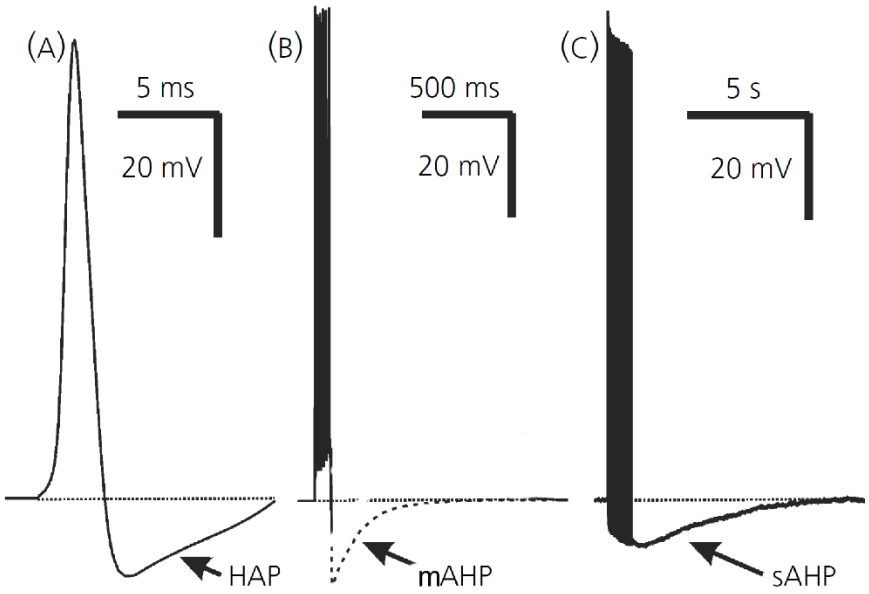

Figure 1.4. Types of after hyperpolarization potentials (AHPs) in MNCs and their contribution to MNC electrical behaviour.
(A) After each individual spike in MNCs there is a prominent postspike hyperpolarizing after-potential (HAP) also called fast after hyperpolarizing potential (fAHP) that is responsible for the keeping the MNCs refractory for approximately 10 ms following the spike. (B) After few multiple spikes in MNCs there is a prominent medium after hyperpolarizing potential (mAHP) that develops and is thought to contribute to spike frequency adaptation during rapid firing events. (C) After prolonged electrical activity in MNCs there is another small amplitude and longer lasting slow after hyperpolarizing potential (sAHP) that develops and is thought to contribute to intraburst frequency and burst duration during burst firing events. The depolarizing after potentials (DAPs) and their effects, which are also present during the MNC spiking, are however not shown here for better understanding of the AHPs. Adapted with permission from (Brown, 2004).

1.3.1.2.1 The fAHP following a single AP spike

The membrane potential of the MNCs after evocation of a single AP spike becomes more negative than the starting resting potential. This after-spike hyperpolarizing potential was termed as the HAP (hyperpolarizing after potential), but is now more commonly referred to as the fAHP (fast afterhyperpolarization potential) (Andrew *et al.*, 1984; Bourque *et al.*, 1985; Roper *et al.*, 2003). The fAHP typically lasts tens of ms and has an amplitude of about 7 mV (Andrew *et al.*, 1984; Bourque *et al.*, 1985). Since the fAHP current is outwardly rectifying and its magnitude and duration was greatly affected by extracellular Ca^{2+} levels (Bourque *et al.*, 1985) it was thought to be mediated by a Ca^{2+}-activated K^+ (K_{Ca}) channel (Andrew & Dudek, 1984; Bourque et al., 1985; Roper et al., 2003). Ca^{2+} activated K^+ (K_{Ca}) channels are a subfamily of the larger K^+ channel family that are gated by changes in intracellular Ca^{2+} levels. K_{Ca} channels are divided into three families based on their biophysical and pharmacological properties: BK, IK, and SK channels (Faber *et al.*, 2003). BK channels are outwardly rectifying K^+ channels and so named because they have big (B) conductance for potassium (K^+) ions. BK channels have a dual activation mechanism and are known to be activated by membrane depolarization and/or by increases in concentration of intracellular calcium ion (Ca^{2+}). Intracellular recordings made in rat MNCs in the presence of pharmacological blockers of BK channels like iberiotoxin have shown that the fAHP could be mediated by BK channels (Greffrath *et al.*, 2004). Another study showed that a transient, Ca^{2+}-dependent outward K^+-selective current that is sensitive to 4-aminopyridine (4-AP; a non-selective blocker of K^+ channels) but not to TEA could also contribute to the HAP and modulate firing rate in the MNCs (Bourque, 1988; Bourque *et al.*, 1998). The rapid biophysical properties of the fAHP suggests that it might be important during the refractory period between successive spikes and thus contribute to development of spike frequency adaptation during rapid firing

events in the MNCs.

1.3.1.2.2 The mAHP and sAHP following multiple AP spikes

Current clamp recordings in hypothalamic explants and slices have shown that multiple AP spikes can evoke the mAHP and sAHP in the MNCs. Both the AHP and the sAHP are activity-dependent currents, which implies that their magnitude and duration are directly dependent on the preceding electrical activity of the MNCs such that both of these AHPs are larger when evoked with a higher frequency AP spike train as compared to when they are evoked with a lower frequency spike train (Kirkpatrick *et al.*, 1996; Roper *et al.*, 2003; Ghamari-Langroudi *et al.*, 2004). Both VP and OT MNCs have been shown to exhibit mAHPs and sAHPs without any significant differences (Armstrong *et al.*, 2010). Both the mAHP and the sAHP are mediated by outwardly rectifying K^+ currents and depend on the Ca^{2+} influx that occurs during electrical activity in neurons, suggesting that they could also be mediated by Ca^{2+}-activated K^+ channels (Ghamari-Langroudi *et al.*, 2004). The mAHP, for example, has been shown to be selectively blocked by apamin and d-tubocurarine (Armstrong *et al.*, 1994; Kirkpatrick *et al.*, 1996; Ghamari-Langroudi *et al.*, 2004), which are pharmacological blockers of small-conductance (SK) channels. SK channels are outwardly rectifying K^+ channels and are so named because they have small (S) single channel conductance for K^+. However unlike the BK channels, SK channels are voltage independent and are only activated by increases in intracellular Ca^{2+}. The mAHP may contribute to the termination of bursts by opposing the DAP and the plateau potential generation in MNCs (Andrew *et al.*, 1984; Bourque *et al.*, 1985). Furthermore, apamin treatment has been shown to inhibit spike frequency adaptation during MNC bursts by increasing the mean intra-burst firing frequency and also shortening the overall burst duration (Kirkpatrick *et al.*, 1996), which suggests that the mAHP might be an important contributor to the bursting behaviour of MNCs.

One of the first studies that investigated sAHP mechanisms in MNCs using SON slices suggested that the sAHP could be mediated by the activation of intermediate-conductance Ca^{2+} activated K^+ (IK) channels (Greffrath *et al.*, 1998). Like BK and SK channels, IK channels also are outwardly rectifying K^+ channels and are so named because their single channel conductance for K^+ is intermediate between the BK and SK channels. Like the SK channels, IK channels are also voltage-independent and are only activated by increases in intracellular Ca^{2+}. Although a later study in hypothalamic explants showed that about 80% of the sAHP in MNCs is due to activation of voltage-independent and Ca^{2+}-dependent K^+ channels (Ghamari-Langroudi *et al.*, 2004), the sAHP was found to be insensitive to IK channel blockers or other standard SK and BK channel blockers and therefore awaits reconciliation with the earlier reports. Although the biophysical properties of the sAHP are consistent with its potential role in burst termination, its physiological role in MNC firing patterns has not been established. This is because the molecular identity of channels mediating the MNCs sAHP is unknown (Ghamari-Langroudi *et al.*, 2004) and therefore channel specific pharmacological modulators could not be used to test the role of sAHP in MNCs firing behaviour. Application of muscarine was found to inhibit the sAHP in hypothalamic explant preparations and increase the burst duration and/or inhibit the bursting behaviour of some MNCs, which suggests that the sAHP could be an important contributor to bursting behaviour of MNCs (Ghamari-Langroudi *et al.*, 2004).

1.3.2 Autocrine control of MNCs

The MNCs are among the first neurons in which evidence for the somatodendritic release of neuropeptides (NPs) was documented and has been extensively studied (Ludwig, 1998). Although VP and OT are the major NPs that are secreted by the MNCs, studies have shown that there are other NPs that are somatodendritically released by them. For example, VP MNCs have been

shown to release NPs like dynorphin, apelin, galanin, neuroendocrine regulatory peptides and secretin while OT MNCs have been found to release dynorphin, proenkephalin A-derived l-opioid peptides and dynorphin (Brown *et al.*, 2013). Although somatodendritic release in MNCs is known to occur independently of axonal release events in the pituitary, the mechanisms underlying the dissociation between the two release systems are not well understood. While the axonal release of MNCs is strictly dependent on electrical activity and requires Ca^{2+} influx through VDCCs, the somatodendritic release occurs both in an electrical activity-dependent and independent fashion that may or may not require Ca^{2+} influx (Ludwig *et al.*, 2006). The dissociation between somatodendritic secretion and axonal release in MNCs has been clearly demonstrated by studies using MSH (melanocyte stimulating hormone) agonists, which stimulate the somatodendritic OT release while inhibiting the systemic release of OT (Ludwig *et al.*, 2006). Another level of complexity that exists in the MNCs autocrine physiology is that the somatodendritic release mechanism and its physiological importance have been better characterized only in VP MNCs (Brown *et al.*, 2013). For example; dynorphin is a k-opioid peptide that is co-localized in the same neurosecretory vesicles as VP and OT along with the k-opioid receptors (Watson *et al.*, 1982; Eriksson *et al.*, 1996; Shuster *et al.*, 2000) but its role in regulating the MNC electrical behaviour is more clear in VP MNCs. Upon fusion of these LDCVs with the dendritic membrane, both VP and dynorphin are released into the SON and the k-opioid receptors are inserted into the dendritic membrane (Shuster *et al.*, 1999). It has been shown that this somatodendritic dynorphin release mechanism in the MNCs provides an efficient autocrine inhibitory feedback mechanism for regulating termination of bursts in VP MNCs (Brown, 2004; Brown *et al.*, 2006). Dynorphin has been shown to inhibit the DAP and the plateau potentials in phasically firing neurons such that in presence of k opioid antagonists, the burst duration and firing frequency of VP MNCs are both

increased. Interestingly the dynorphin-mediated burst termination effect is activity-dependent implying that the inhibitory effect is absent at the beginning of bursts but develops slowly during the burst progression. Though the co-released VP also has an inhibitory effect on the MNC firing rate, the effect of VP is activity-independent and occurs in a more tonic fashion in comparison to dynorphin (Brown *et al.*, 2006).

Another NP somatodendritically-released by MNCs is apelin, which is stored in different vesicles and is not present alongside VP (Reaux-Le Goazigo *et al.*, 2004). The presence of apelin in different vesicles raises the possibility that its release can be independently regulated from VP release; the physiological significance and role of which however awaits further studies. Since centrally-administered apelin has been shown to inhibit the activity of VP MNCs (De Mota *et al.*, 2004) while the local administration of apelin into the SON was shown to excite VP MNCs (Tobin *et al.*, 2008), the true effect of apelin on VP MNC activity is still debated. Galanin, another NP secreted by VP MNCs has also been proposed to have an autocrine inhibitory effect on VP MNCs that might be important for keeping a check on the over-excitation during demanding conditions (Brown *et al.*, 2013). VP MNCs also secrete pituitary adenylate cyclase-activating peptide (PACAP), which has been shown to exert a direct excitatory effect on MNCs (Shibuya *et al.*, 1998a) and also shown to inhibit them indirectly by stimulating somatodendritic release of VP (Shibuya *et al.*, 1998b). It remains to be seen how the two opposing effects of PACAP would interact under different physiological conditions to affect the overall VP MNC activity.

Finally the somatodendritic release of VP and OT has itself been proposed to regulate MNC activity in the SON in a complex fashion. For example, local administration of exogenous VP in the SON has been shown to inhibit the VP MNCs that are firing in phasic patterns or continuous firing modes but have been shown to excite the VP MNCs that are firing irregularly or are

relatively silent (Gouzenes *et al.*, 1998). Similarly exogenous administration of OT in SON has been shown to stimulate the OT MNC activity by promoting the bursting activity during milk-ejection reflexes (Moos *et al.*, 1989), while it has also been shown to inhibit excitatory post synaptic currents (EPSCs) in MNCs by triggering the release of endocannabinoids from the postsynaptic MNC (Kombian *et al.*, 1997; Hirasawa *et al.*, 2004), which would be expected to make OT MNCs less excitable.

1.4 Osmotic mechanisms regulating MNC physiology

Jewell and Verney first proposed in the 1950's that neurons regulating the osmotically-evoked VP release might be located in the SON (Jewell *et al.*, 1957). This was later confirmed by immunohistochemical studies that demonstrated that MNCs located in the SON and PVN of the hypothalamus are indeed the major VP and OT releasing hormones in the hypothalamus (Swaab *et al.*, 1975; Vandesande *et al.*, 1975). Many independent studies in the past have shown that MNCs are activated by hypertonic challenges and inhibited by hypotonic conditions and that they could regulate the systemic levels of VP and OT in accordance with the prevailing osmotic conditions (Walters *et al.*, 1974; Brimble *et al.*, 1977; Brimble *et al.*, 1978; Wakerley *et al.*, 1978; Neumann *et al.*, 2008). However it was not clear during these experiments if the activation of MNCs and the subsequent release of osmoregulatory hormones was due to their direct osmosensing properties or due to signalling from other osmosensitive regions in the brain or a combination of both factors. Therefore to ascertain the true mechanism of MNC osmosensitivity, patch clamp recordings under a variety of experimental conditions have been performed in hypothalamic explants, SON slices and acutely isolated MNCs as described below.

1.4.1 Osmosensitive stretch-inactivated channels (SICs)

The intrinsic osmosensitive properties of MNCs were first proposed after intracellular recordings

made in hypothalamic slices showed that the SON MNCs could depolarize upon hypertonic stimulation under conditions of blocked synaptic transmission (Mason, 1980). However the true osmosensitivity of MNCs was first demonstrated using whole cell patch clamp technique on acutely isolated MNCs (a preparation devoid of all synaptic or glial inputs on MNCs). Using voltage ramp current protocols, it was found that isolated MNCs could depolarize and exhibit an increase in their non-specific ion conductance when challenged with an acute hypertonic stimulus (Oliet *et al.*, 1992; Oliet *et al.*, 1993b; Oliet *et al.*, 1993a). Since a similar increase in ion channel conductance could also be evoked by application of pressure in the cell-attached patch pipette and/or hypertonic stimulation of the isolated MNCs, it was proposed that the intrinsic osmosensitivity of MNCs could be due to the presence of mechanosensitive channels whose opening probability depends on amount of tension present in their membrane (Oliet *et al.*, 1993a). Studies by the same group have shown that increases in cellular conductance evoked by acute hypertonic stimulation of the isolated MNCs could be reversed by application of positive pressure in whole cell patch pipettes (Zhang *et al.*, 2007b), which confirms that both osmotic stimulation and the patch pipette pressure are modulating the same ion channels.

Since the MNCs were hyperpolarized (due to a decrease in an inward cationic conductance) by hypotonic stimulation (Oliet *et al.*, 1993a; Oliet *et al.*, 1993b) or by application of positive pressure in the patch pipettes that would cause swelling of MNCs even in the absence of hypotonic stimulation thereby causing stretching of MNCs membrane (Zhang *et al.*, 2007b), the osmosensory mechanosensitive channels are more commonly referred to as SICs (stretch-inactivated channels). The osmotically-evoked increase in MNC firing was significantly inhibited in the presence of Gd^{3+} (which is a non-specific blocker of SICs (Oliet *et al.*, 1996)), suggesting that the depolarizing excitatory trigger provided by the osmotic activation of SICs also contributes to

their increased electrical activity during hypertonic conditions. The basic mechanism of SIC activation and its contribution to MNC electrical activity is summarized in Figure 1.5.

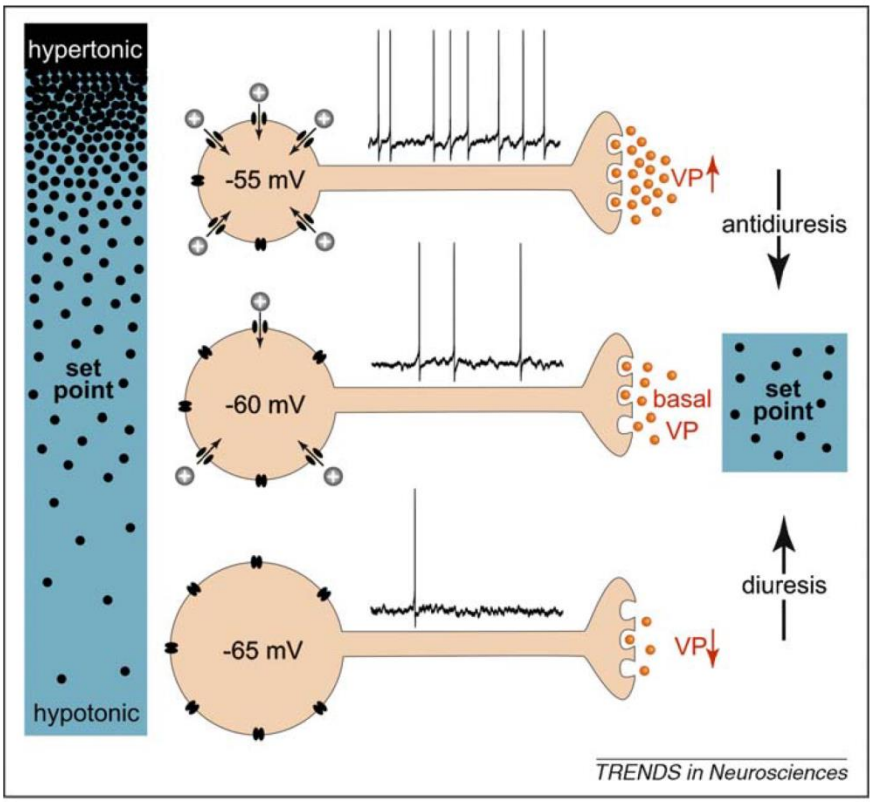

Figure 1.5. Osmosensitive SICs and their contribution to MNC electrical activity.
Changes in osmolality cause inversely proportional changes in MNCs volume and the opening probability of stretch-inactivated channels (SICs) in their membranes. For example hypertonicity causes MNC shrinkage that activates the SICs. The increased opening of SICs causes MNCs depolarization and facilitates an increase in their AP firing rate. This allows MNCs to increase their systemic release of vasopressin (VP) and oxytocin (OT) which can then act on kidneys to cause antidiuresis and natriuresis (not shown) both of which are physiological processes that restores the increased osmolality. Adapted with permission from (Prager-Khoutorsky *et al.*, 2010).

Current-voltage analysis of cell attached recordings has shown that the open channel conductance of the SIC is between 30-35 picosiemens (Oliet *et al.*, 1993a), while the whole cell recording using voltage ramps have shown that osmotically-evoked SIC activation is mediated by an increase in non-specific cation conductance that normally has a reversal potential between -30 to -40 mV under physiological conditions (Oliet *et al.*, 1993a; Oliet *et al.*, 1993b; Ciura *et al.*, 2011). Although SIC currents were initially thought to be mediated by a non-specific K^+ and Na^+ conductance (Oliet *et al.*, 1993a), a later study by the same laboratory showed that SICs are also highly permeable to Ca^{2+} (Zhang *et al.*, 2006), which suggests that under normal physiological conditions, the osmotically-evoked SIC conductance is mediated by all the three major cations that are present in the biological fluids.

Recent studies have demonstrated that the SICs mediating osmosensitivity in MNCs could be encoded by an N terminal variant of the transient receptor potential vanilloid type-1 gene (TRPV1) that is insensitive to capsaicin activation (Sharif Naeini *et al.*, 2006; Zaelzer *et al.*, 2015). It was found that MNCs of TRPV1 knockout mice did not exhibit the increase in cation conductance upon hypertonic stimulation, although their cellular shrinkage was similar to that observed in the wild type MNCs. It has also been shown that the osmotic activation of SICs in wild type MNCs is significantly reduced in the presence of ruthenium red (RuR), which is non-specific TRPV blocker (Sharif Naeini *et al.*, 2006). The role of TRPV1 in the osmotic physiology of MNCs was further supported by observations that TRPV1 knockout animals had defects in systemic osmoregulation as these animals showed a lower increase in their systemic VP levels during chronic hypertonic conditions in comparison to wild type mice (Sharif Naeini *et al.*, 2006). Although the exact composition of the SIC channel in MNCs, whether it is heteromeric or homomeric or if it needs specific auxiliary subunits, is not known (Sudbury *et al.*, 2010), a recent study has shown that

expression of the N-terminal variant of TRPV1, but not the wild type TRPV1, could rescue the osmosensory response in hypothalamic neurons obtained from TRPV1 knockout mice (Zaelzer *et al.*, 2015). This study, therefore, suggest that N-terminal variant of TRPV1 probably plays the most important role in mediating the osmotic conductance in MNCs.

Although the mechanosensitive nature of SICs was first found in the 1990s, the basis of their mechanosensitivity is only becoming clear now. The mechanosensitive properties of SICs have now been attributed to the presence of a uniquely-arranged scaffold of microtubules (Prager-Khoutorsky *et al.*, 2014) in the MNC somata and the presence of an intact actin cytoskeleton in the cell periphery (Zhang *et al.*, 2007b), both of which have been shown to respond to the volumetric changes occurring in the MNCs during osmotic treatments. Using patch clamp methods it was shown that the osmotic activation of SICs was greatly enhanced in the presence of jasplakinolide, a drug that promotes actin polymerization, whereas the osmotic activation of the SICs was greatly reduced when MNCs were pre-treated with cytochalasin-D, a drug that disrupts actin microfilaments inside the cells. The actin polymerization mechanism that contributes to osmotic activation of SICs in MNCs has also been shown to contribute to angiotensin II-mediated activation of SICs activity through a G-protein coupled receptor (GPCR) linked PKC-dependent process (Zhang *et al.*, 2007b). Although the true mechanism by which actin filaments interact with and gate the SICs is currently unknown, the fact that both osmotic and GPCR-evoked pathways caused actin polymerization suggest these two pathways might be utilizing overlapping intracellular signalling mechanisms to increase the SIC activity.

Using whole cell patch clamp techniques on acutely isolated MNCs, it was also shown that osmotic activation of SICs in MNCs was greatly enhanced in the presence of taxol, a drug that stabilizes microtubules, whereas it was significantly prevented in the presence of nocodozole, a

compound that disrupts microtubule structures. Contrary to actin filaments, the microtubules have been shown to physically associate with the C terminal of TRPV1 encoded SICs in the MNC membrane. Using sequence-specific synthetic peptides to block the potential binding sites between TRPV1 and tubulin molecules, the researchers were further able to prevent the osmotic and mechanically-evoked activation of SICs thereby suggesting that a direct pushing force mediated by physical association of TRPV1 and microtubules, at specific interaction sites, is indeed the underlying mechanism of this activation (Prager-Khoutorsky *et al.*, 2014). It is however important to note that the TRPV1 and microtubules association is not affected by angiotensin-mediated GPCR signalling, which suggest the TRPV1 and microtubule association is independent of GPCR signalling.

1.4.2 Osmotically-modulated neuronal and astrocytic transmission in the SON

Although MNCs are intrinsically osmosensitive, they also receive synaptic inputs from neighbouring intrinsically osmosensitive brain regions like the OVLT (vascular organ of lamina terminalis), the MnPO (median pre-optic nucleus) and the SFO (subfornical organ) that affect the electric behaviour of MNCs (Brown *et al.*, 2013; Armstrong *et al.*, 2014). Similarly the MNCs also receive processes from surrounding osmosensitive astrocytes that are capable of detecting and responding to osmotic changes (Choe *et al.*, 2012). The MNCs perceive this osmotic information from nearby brain regions the form of various neuromodulators such as glutamate, GABA (gamma-aminobutyric acid) and angiotensin, whereas they receive this information from the nearby astrocytes in the form of taurine. The synaptic innervation from the OVLT and SFO are especially important because both the OVLT and SFO are circumventricular organs (CVOs), which means they are located at sites surrounding the ventricular system of the brain and are outside the blood brain barrier (BBB). This strategic anatomical location allows them to detect changes in

the plasma osmolarity more quickly than the MNCs and then facilitate the MNCs response through their synaptic inputs.

1.4.2.1 Osmotically-modulated synaptic inputs onto MNCs

The OVLT neurons have mechanosensitive TRPV1 channels like the MNCs and thus they are directly activated by volume changes evoked by increases in plasma osmolality (Ciura *et al.*, 2006; Ciura *et al.*, 2011). The SFO astrocytes have Na^+ sensing (Na_X) channels that are activated by sensing increases in plasma Na^+ levels and can in turn modulate the activity of SFO neurons (Noda *et al.*, 2015). The MnPO neurons have also been shown to have intrinsic Na^+ sensing capabilities (Grob *et al.*, 2004) and thus contribute to osmosensory functions in the brain (Maughan *et al.*, 2001; McKinley *et al.*, 2003). Previous studies have shown that local intracerebral infusion of hypertonic solutions or electrical stimulation of these brain regions can also activate MNCs (Richard *et al.*, 1995; Brown *et al.*, 2013; Armstrong *et al.*, 2014), which suggests that there are excitatory synaptic afferents that carry electrical information from these regions to the MNCs. However, there also have been reports suggesting the presence of inhibitory inputs on the MNCs from these brain regions, the role of which are not well understood. For example, both the excitatory (glutamergic) and inhibitory (GABAergic) inputs of MNCs have been shown to increase during chronic hypertonic conditions (Di *et al.*, 2004) but only the glutamergic inputs are known to increase during acute hypertonic stimulation of OVLT (Richard *et al.*, 1995). Although the increased glutamergic inputs during chronic hypertonic conditions are apparently relevant for sustained increases in MNC activity, it is unclear how increased GABAergic inputs could facilitate MNC exciatation. One possibility that has been suggested for this is differential regulation of glutamergic and GABAergic MNCs inputs by norepinephrine whose release in SON is also increased during chronic hyperosmotic conditions. It has been proposed that norepinephrine has

a facilitatory effect on glutamate but inhibitory effect on GABA release, which allows it to compensate the increased inhibitory inputs and thus tip the balance in favour of excitatory controls (Di *et al.*, 2004). Another possible reason for increased GABA inputs could be their excitatory nature because it has been shown that GABA inputs to VP MNCs in adult rats are excitatory due to lack of expression of the K^+-Cl^- cotransporter 2 (KCC2), which is the predominant Cl^- exporter in the adult brain, thereby causing a reversal of the normal Cl^- equilibrium potential (Haam *et al.*, 2012). An increase in excitatory GABA inputs could therefore be a physiological adaptation to facilitate increased VP MNC activity during hyperosmotic stress. Another study has shown that although GABA inputs to the MNCs are inhibitory under normal isotonic conditions, they become excitatory during conditions of hyperosmotic stress. This switch is facilitated by an increase in expression of Na^+-K^+-$2Cl^-$ cotransporter 1 (NKCC1) in MNCs, which is the predominant Cl^- importer in the early stages of brain development, thereby causing an accumulation of intracellular Cl^- and directly contributing to the activation of MNCs, much like conventional glutamergic inputs (Kim *et al.*, 2011b). The presence of these complex excitatory and inhibitory synaptic mechanisms and their modulation could be an intrinsic mechanism that prepares MNCs to transduce the osmotic perturbations in a more graded and controlled manner without causing over-excitation of the MNCs. Although a general consensus over the nature and functional significance of GABA inputs to the MNCs has not yet been reached, the plastic and functional changes in overall synaptic innervations on the MNCs that occurs during osmotic perturbations seem important in modulating the MNCs activity and overall osmoregulation in the body.

Although the exact nature of excitatory synaptic inputs from SFO neurons has not been established it has been shown that at least some part of this excitatory transmission could be mediated by angiotensin by its direct action on the metabotropic receptors present in the MNCs

(Jhamandas *et al.*, 1989; Armstrong *et al.*, 2014). Lesions in both the SFO and the MnPO regions have been shown to inhibit the osmotically-evoked increase in MNC electrical activity (Brown *et al.*, 2013), which suggests that constant inputs from these regions do contribute to osmotic physiology of MNCs, the neurotransmitters mediating this effect has not been identified. It has been shown for example, that lesions in the MnPO region could reduce the osmotically-evoked secretion of VP in the posterior pituitary (Leng *et al.*, 1989; Brown *et al.*, 2013) and that osmotic stimulation of the MnPO directly excites the SON neurons (Honda *et al.*, 1990), suggesting that there might be direct excitatory control of MnPO over the MNCs. However it has also been shown that focal electrical and chemical stimulation of the MnPO could inhibit the MNC activity in a GABA-dependent fashion (Nissen *et al.*, 1994), which suggests that the MnPO might have an inhibitory control over MNC activity. Regardless, the fact that there is more than one osmotic centre in the brain suggests there is an osmoregulatory network in the brain that is constantly protecting us against osmotic insults.

1.4.2.2 Osmotically-modulated changes in gliotransmission on to MNCs

Although MNCs are the major neuronal population in the SON, there are other non-neuronal cell types present in the SON, such as the microglia and astrocytes (Ayoub *et al.*, 2003; Blumenstein *et al.*, 2004). Over the last two decades there has been a significant shift in our understanding of the role of non-neuronal cells, especially astrocytes, in regulating neuronal function. Astrocytes, which were once believed to the silent structural supporters in the brain, are now being increasingly appreciated as modulators of neuronal function, which they do by responding to neurotransmitters released by neurons and also talking back to neurons by releasing gliotransmitters such as adenosine triphosphate (ATP), glutamate, D-serine and taurine (Volterra *et al.*, 2005; Santello *et al.*, 2012; Harada *et al.*, 2015). SON astrocytes have been shown to send

processes to MNCs that are usually seen wrapped around the MNCs under normal conditions. However chronic hyperosmotic conditions and lactating conditions have been shown to cause retraction of the astrocyte processes from the MNCs in immunohistochemical studies thereby suggesting that astrocytic process might have a role in modulating the physiology of MNCs (Tweedle *et al.*, 1976; Stern *et al.*, 1998; Brown *et al.*, 2013).

Patch clamp recording experiments have shown that hypo-osmotically activated channels in SON astrocytes release taurine, which has an inhibitory control over the MNCs. Specifically, taurine has been shown to activate Cl^--permeable glycine receptors present on the MNCs membrane, which hyperpolarizes the MNCs and thus inhibits their activity (Hussy *et al.*, 1997; Hussy, 2002; Choe *et al.*, 2012). During hypertonic conditions, the cellular shrinking in astrocytes inhibits these hypo-osmotically activated channels and thereby prevent their taurine release. The absence of taurine during hypertonic conditions therefore relieves the glycine receptor-mediated inhibitory control over the MNCs and this contributes to the activation of MNCs. Since astrocytes also have glutamate transporters and are often involved in clearing the synaptic glutamate levels, the retraction of astrocytic processes during chronic hypertonic and lactating conditions is ideal for creating a taurine-deficient and glutamate-rich environment near MNCs (Oliet *et al.*, 2001), which could allow better and prolonged activation of MNCs and is therefore likely to be an important physiological adaptation.

Although the taurine-mediated osmotic regulation of MNC activity by astrocytes has been extensively studied (Hussy *et al.*, 1997; Choe *et al.*, 2012) there are potentially other mechanisms by which astrocytes could modulate MNC behaviour. For example, osmotic activation of astrocytes cultured from other parts of brain have been shown to secrete ATP upon hypotonic swelling through maxi-anion channels (Liu *et al.*, 2008). Although the adrenergic stimulation of

SON astrocytes has been shown to enhance MNC activity by facilitating the increase in AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid) receptor density in the MNC membranes through purinergic signalling mechanisms (Gordon *et al.*, 2005), it is unknown if osmotic activation of SON astrocytes could also activate this pathway by stimulating ATP release. Moreover the ATP released by SON astrocytes in response to presynaptic glutamate that reaches the SON from other brain regions (Gordon *et al.*, 2009) could also activate AMPA receptor trafficking and thus facilitate the electrical activity of MNCs. Astrocytes are also known to release D-serine, which acts as a co-agonist at post-synaptic NMDA (N-methyl-D-aspartate) receptors in MNCs and thus contributes to their electrical behaviour and also to the hypothalamic plasticity in the SON under different physiologically demanding conditions (Panatier *et al.*, 2006). Astrocytes have also been shown to release IGF (insulin growth factor), which acts to further decrease the electrical activity of MNCs by a dual mechanism that involves inhibition of depolarizing SICs currents and potentiation of hyperpolarizing glycine receptor (GlyR) mediated Cl^- currents (Ster *et al.*, 2005). The mechanisms causing the astrocytes to release IGF1 are however unknown. Finally astrocytes in SON have been shown to express both glutamate (Boudaba *et al.*, 2003) and GABA transporters (Park *et al.*, 2006) in their membranes, which suggests that they are directly involved in synaptic clearing of these neurotransmitters and could thus indirectly contribute to MNC electrical behaviour.

1.4.3 Other osmosensitive currents

MNCs are also known to have another functional K^+ channel that mediates hyperpolarizing outward currents in an osmotic fashion (Zhang *et al.*, 2009). Patch clamp recordings in acutely isolated MNCs have shown that acute hypertonic stimulation can cause activation of a slowly activating and inactivating potassium current called the OKC (osmosensitive K^+ current) that

belongs to K_V7 family and is TEA and 4-AP insensitive and is partially Ca^{2+} dependent. The role of the OKC is not completely understood since an outward K^+ current that is activated under hypertonic conditions would be expected to cause MNC hyperpolarization rather than depolarization, which is characteristic behaviour of MNCs under hypertonic conditions. MNCs also have stretch-activated K^+ channels in their membranes including members of TASK (TWIK-related acid-sensing K^+ currents), TREK (TWIK-related K^+ currents) and TRAAK (TWIK-related arachidonic acid activated K^+ currents) families, which are apparently sensitive to mechanical factors or extracellular pH but are voltage insensitive (Talley *et al.*, 2001; Han *et al.*, 2003). Since their opening probability was found to increase during membrane stretching, they could potentially contribute to the hyperpolarizing response of MNCs during hypotonic conditions. The molecular identities, activation mechanisms and physiological roles of these channels and also the channels mediating OKC in regulating the osmotic physiology of MNCs is not clear at this time and awaits further studies. The slowly activating and inactivating properties of the OKC however suggest that it could contribute to burst termination in MNCs, which is an important adaptation of VP MNCs during conditions that demand maximum VP release.

1.4.4 Osmotically-modulated peripheral inputs onto MNCs

Besides the central osmoreceptors, there also are peripheral osmoreceptors that are located outside the brain, which detect osmotic changes and contribute to body fluid balance. The most well-known peripheral osmoreceptors are located in the oropharyngeal cavity, the splanchnic mesentery and the thoracic dorsal root ganglia that innervate hepatic blood vessels (Bourque, 2008; Lechner *et al.*, 2011). The peripheral osmoreceptors are mostly located on the luminal side of the alimentary tract and the blood vessels linings that are directly involved in absorption and transport of ingested materials. The peripheral osmoreceptors are important because they are

the first to encounter the ingested food and drinks and sense their potential osmotic impact in the body. For example, it has been shown that gastric salt loading can increase the osmolarity of blood in the hepatic portal vein within 7 minutes, whereas increase in overall plasma osmolarity is detected only after about 15 minutes (Carlson *et al.*, 1997). Although the afferent pathways linking the peripheral osmotic centre to the MNCs are not very clear, the MNCs are thought to receive noradrenergic excitatory synaptic inputs from the NTS and ventrolateral medulla, which carry and relay the osmotic information from the peripheral osmoreceptors to the MNCs (Onaka *et al.*, 1995; Brown *et al.*, 2013). Although peripheral osmoreceptors are important for detecting the osmotic changes in response to our drinking and eating behaviour, their cellular mechanisms and molecular structures are less clearly understood in comparison to their central counterparts (Bourque, 2008; Lechner *et al.*, 2011).

1.5 The Phospholipase C signaling pathway and its effector molecules

Biological plasma membranes are semipermeable hydrophobic barriers that are essentially made of proteins, carbohydrates and lipids (Khurana, 2014b). The major function of the plasma membrane is to regulate the transport of substances into and out of the cells and also help maintain their structural integrity. The hydrophobicity and selectively permeability of the plasma membrane are due to the bilayer (two layers) arrangement of phospholipids (one of the type of membrane lipids that are amphipathic in nature and have an esterified phosphate group in their chemical structure) such that the hydrophobic "tails" of phospholipid molecules in each layer are facing away from the aqueous environment present on either side of the cell membrane while their hydrophilic "heads" are respectively facing towards the intracellular and extracellular sides (Sembulingam *et al.*, 2011; Khurana, 2014b).

Phosphoinositide-specific phospholipase C (PLC) is a key intracellular enzyme that upon activation, hydrolyzes phosphatidylinositol 4, 5-bisphosphate (PIP_2), a membranous phospholipid and generates two second messengers: diacylglycerol (DAG) and inositol 1, 4, 5-trisphosphate (IP_3). DAG, which remains in the membrane, activates protein kinase C (PKC), whereas IP_3, which diffuses into the cytoplasm, triggers the release of Ca^{2+} from intracellular stores. Both these effects have been shown to regulate a wide variety of intracellular functions (Garrett *et al.*, 2013; Kadamur *et al.*, 2013). PLC enzymes transduce extracellular signals into an intracellular signalling cascade that initiates appropriate response by the cellular machinery. Although PIP_2 is only a minor membrane phospholipid whose concentration is usually less than 1% of total phospholipids present in the membrane, it is an important signalling molecule and is the major precursor for DAG and IP_3 (McLaughlin *et al.*, 2002). The diversity of PLC-mediated PIP_2 signalling pathways in mediating different intracellular functions is discussed in the following sections.

1.5.1 PLC isoforms

Thirteen mammalian PLC isozymes have been identified and grouped into six different families based on their sequence homology and domain structure (Suh *et al.*, 2008b; Kadamur *et al.*, 2013). The six families with the known isoforms in brackets are; PLC β (PLC β1, β2, β3 and β4), PLC γ (PLC γ1 and γ2), PLC δ (PLC δ1, δ3 and δ4), PLC ε (PLC ε), PLC ζ (PLC ζ) and PLC η (PLC η1 and η2). All of these isoforms express two highly conserved catalytic domains called X and Y domains that are formed of alternating α-helices and β-strands and are mainly responsible for their PIP_2 hydrolyzing action (Suh *et al.*, 2008b; Kadamur *et al.*, 2013). The highest PLC expression has been found in the brain, which suggests that PLC mechanisms are important in neuronal signaling (Suh *et al.*, 2008b). Since most of the PLC enzymes have also been found to have multiple splice variants, a total of more than 30 different PLC isoforms are now known to exist in mammals (Suh *et al.*, 2008b).

Although all PLC isoforms cleave membrane PIP_2 in a similar fashion and have a conserved catalytic domain that includes a calcium binding motif, there is significant diversity in their activation mechanisms and this is likely to contribute to their tissue-specific responses to different physiological stimuli. For example, while activation of members of the PLC β and PLC γ families are specifically associated with ligand-mediated stimulation of G-protein coupled receptors (GPCRs) and receptor tyrosine kinases (RTKs) respectively, PLC ε responds to both GPCR and RTK stimulation and the activation mechanism of PLC ζ is currently unknown (Suh *et al.*, 2008b). On the other hand, despite the fact that all PLC isoforms require intracellular Ca^{2+} to exhibit full activation, only members of PLC δ (Allen *et al.*, 1997; Kim *et al.*, 1999) and PLC η (Nakahara *et al.*, 2005; Kim *et al.*, 2011a) families have been shown to activate in response to increased Ca^{2+} concentrations, which suggests that an increase in Ca^{2+} binding is a sufficient trigger for

activation of these PLC isoforms. Since receptor-mediated activation of PLC β, PLC γ and PLC ε isoforms also results in increases in intracellular Ca^{2+} levels through IP_3-mediated opening of intracellular Ca^{2+} stores, the Ca^{2+}-mediated activation mechanism of PLC δ and PLC η isoforms could also amplify the PLC signaling pathways of other isoforms. Moreover, as increases in intracellular Ca^{2+} can be achieved by a variety of other non-receptor linked physiological means (for example by TRPV1 agonists (Lukacs *et al.*, 2013; Borbiro *et al.*, 2015), mechanical stress (Ryan *et al.*, 2000), heat or cold treatment (Daniels *et al.*, 2009), depolarization of the cell membrane (Kendall *et al.*, 1985; Lukacs *et al.*, 2013) and hyper-osmotic treatment (Takahashi *et al.*, 2001; Komis *et al.*, 2008)), the PLC η and δ isoforms could be activated in response to a host of physiological stimuli.

1.5.2 The role of PIP_2 signalling in cellular physiology

The PIP_2 signalling pathway is activated by the action of PLC on membrane PIP_2 and encompasses three signalling molecules (PIP_2, DAG and IP_3) each of which is capable of triggering intracellular processes independently or in combination with other signalling molecules (Kadamur *et al.*, 2013). PIP_2 signalling pathways have been shown to play key roles in proliferation, differentiation, apoptosis, cytoskeleton remodelling, vesicular trafficking, chemotaxis, cell migration and neurotransmission, all of which are key processes for normal biological growth and survival (Suh *et al.*, 2008b; Spadaro *et al.*, 2013). Genetic silencing of some of the PLC isoforms has been found to produce knockout mice that have characteristic phenotypes. For example, PLC δ1 knockout mice have been shown to have significant hair loss due to abnormal hair follicle structures. These mice also have marked skin inflammation and epidermal hyperplasia, which suggests the importance of the PL δ1 isoform in the maintenance of skin immune system and hair growth (Ichinohe *et al.*, 2007; Nakamura *et al.*, 2008). PLC- β1 knockout mice have been shown to

develop schizophrenia-like behaviour, which suggests a role of this isoform in normal cortical development (McOmish *et al.*, 2008). PLC- β4 knockout mice have abnormal visual processing, suggesting the importance of PLC- β4 in the vision system (Jiang *et al.*, 1996). Although PLC γ1 knockouts are embryonically lethal (Ji *et al.*, 1997), a conditional knockout of PLC γ1 prepared by using embryonic stem cells showed that this isoform is crucial for normal functioning of the kidney (Shirane *et al.*, 2001). Knockout of PLC δ3 isoforms has yielded phenotypes that have impaired microvilli formation in enterocytes and radial migration of neurons in the cerebral cortex (Nakamura *et al.*, 2013), whereas PLC δ4 knockouts have higher chances of developing sterility (Fukami *et al.*, 2001). In summary, PLC enzymes have been shown to regulate a diverse range of physiological functions and therefore have important roles in cellular physiology.

1.5.3 Roles of PLC signaling in regulating ion channel behaviour

PIP_2 signalling is particularly important for the regulation of the electrical behaviour of excitable cells (Hilgemann *et al.*, 2001). This particular function is largely mediated by the ability of PIP_2 molecules to interact with ion channels and transporters (Hilgemann *et al.*, 2001; Suh *et al.*, 2008a). Although the exact mechanisms by which PIP_2 interacts with these membrane proteins are still being debated, one model suggests that PIP_2 can directly bind to them in a physical manner whereas the other model suggests that PIP_2 can modulate them by electrostatic attractions caused by its negatively charged head groups (Suh *et al.*, 2008a). It has been further proposed that the actual allosteric modulation of ion channel gating by PIP_2 actions might fall somewhat between these two extreme models (Suh *et al.*, 2008a).

Nevertheless, PIP_2 regulates many different ion channels such as the ones belonging to the TRP and K^+ channel families. PIP_2 has been shown to both inhibit and activate ion channels. For example, application of exogenous PIP_2 on excised patches has been reported to increase the

activity and prevent the rundown of many ion channels such as $K_V2.1$ (Hilgemann *et al.*, 2001) $K_V1.1$ and $K_V3.4$ (Oliver *et al.*, 2004), K_{ATP} (Hilgemann *et al.*, 1996), TRPV1 (Stein *et al.*, 2006), $TRPM_7$ (Runnels *et al.*, 2002), Kir_3 (Keselman *et al.*, 2007) and K_V7 (Li *et al.*, 2005) whereas an indirect inhibitory effect has been observed for channels like EAG1 (Han *et al.*, 2016), TRPA1 (Kim *et al.*, 2008) and TRPC4 (Otsuguro *et al.*, 2008). The role of PIP_2 in regulation of ion channel behaviour in intact cell systems has similarly been tested by including exogenous PIP_2 in whole cell recording pipettes (Albert *et al.*, 2008; Thyagarajan *et al.*, 2008; Zhang *et al.*, 2013; Takahashi *et al.*, 2014) or by using treatments that can alter the endogenous levels of membrane PIP_2, for example by GPCR mediated decreases in membrane PIP_2 levels (Zhang *et al.*, 2003a; Li *et al.*, 2005; Brown *et al.*, 2007). Although the effect of PIP_2-mediated regulation for most ion channels for example K_{ir} and K_V7 is now well established, there are conflicting reports that have demonstrated that PIP_2 could exert both stimulatory and inhibitory effects on TRPV1 channel activity (Rohacs *et al.*, 2008; Rohacs, 2015).

It is also important to note that direct interaction between membrane PIP_2 and ion channels is not the only manner by which PIP_2 could regulate ion channel functioning. Downstream signalling molecules, especially DAG also influences ion channel gating and function. Since DAG generation activates PKC, which is known to regulate the function of various intracellular proteins by phosphorylating them, PLC activation could potentially modulate ion channel function in both a direct (PKC-independent) and an indirect (PKC-dependent) manner. The direct effect DAG on ion channels has been demonstrated by exogenous application of DAG analogues both in intact cells and excised patches containing TRPC3 and TRPC6 channels (Hofmann *et al.*, 1999), TRPC2 (Lucas *et al.*, 2003), TRPC7 (Beck *et al.*, 2006) and TRPV1 channels (Woo *et al.*, 2008). DAG mediated activation of PKC also modulate the function of many ion

channels (Shearman *et al.*, 1989). For example, PKC stimulation can activate K_{ATP} (Light *et al.*, 2000), BK (Barman *et al.*, 2004) and TRPV1 channels (Studer *et al.*, 2010; Li *et al.*, 2014) in both excised patches and intact cells. It has also been shown to inhibit the activity of K_{ir} channels (Keselman *et al.*, 2007). There have been reports suggesting that PIP_2 and its hydrolysis products can act together to reinforce a similar effect on ion channels (Keselman *et al.*, 2007) or they can act in an opposing manner such that the net effect on the channel is governed by the extent of the activation of the PLC signalling pathway (Lukacs *et al.*, 2013).

Finally, there have been studies suggesting that PKC could also trigger translocation of pre-existing ion channel-containing vesicles from the cytoplasm to the cell membrane, which may contribute to sustained changes in the electrical behaviour of cells. For example, PKC activation causes translocation of Ca^{2+} channels in molluscan neuroendocrine cells (Strong *et al.*, 1987) and of TRP channels in expressed cell systems (Morenilla-Palao *et al.*, 2004). PIP_2-mediated regulation of ion channel function is therefore variable and depends on the signalling molecules that are involved, the ion channel being studied, the cell type in which the interaction takes place and the time period over which the effects are being studied.

1.5.4 PLC signalling in MNCs

The role of intracellular PLC signalling events in MNC physiology is not well understood and has been mostly studied in context of GPCR-evoked regulation of MNC physiology. For example, previous studies have shown that GPCR agonists such as muscarine (Ghamari-Langroudi *et al.*, 2004), cholecystokinin, neurotensin, and angiotensin II (Yang *et al.*, 1992; Chakfe *et al.*, 2000), affect the MNC electrical behaviour. Although the identity of ion channels underlying the muscarine-mediated increase in MNC firing frequency is not clear (Ghamari-Langroudi *et al.*, 2004), cholecystokinin, neurotensin and angiotensin II have been shown to cause MNC excitation

by increasing the SIC conductance in isolated MNCs (Chakfe *et al.*, 2000; Zhang *et al.*, 2008).

Four different PLC isoforms (PLC β4, PLC δ1, PLC δ4 and PLC γ1) have been detected in SON using DNA microarrays (Hazell *et al.*, 2012) but the exact role and activation mechanisms of these isoforms are unknown. Since MNCs express both GPCRs (Hazell *et al.*, 2012) and RTKs (Arancibia *et al.*, 2007; Carreno *et al.*, 2011) that are commonly linked with PLC β and PLC γ signalling pathways (Suh *et al.*, 2008b; Garrett *et al.*, 2013), it is possible that either PLC β or PLC γ or both isoforms might be involved in transducing receptor-evoked PLC actions in the MNCs. MNCs also exhibit significant increases in their intracellular Ca^{2+} levels by osmotically-evoked opening of SICs channels (Zhang *et al.*, 2006), which could lead to depolarization-mediated Ca^{2+} influx through activation of various voltage dependent calcium channels (VDCCs) that are expressed in MNCs (Fisher *et al.*, 1996), it is therefore tempting to speculate that one or both of the PLC δ isoforms might be activated in response to osmotically-evoked intracellular Ca^{2+} increase in MNCs. PLC δ isoforms could also be activated by intracellular Ca^{2+} increases caused by receptor-evoked PLC-IP_3 signalling by other PLC isoforms and thus contribute in amplifying their response in MNCs. Alternatively, the increase in electrical activity which causes increase in intracellular Ca^{2+} and activation of K_{Ca} channels as discussed before, could also activate these Ca^{2+} sensitive PLC isoforms and thus allow them to contribute in regulation of firing patterns of MNCs. The identification of exact physiological role and activation mechanisms of all these PLC isoforms in MNCs awaits further studies.

1.6 Slack and Slick proteins and Na^+-activated K^+ channels

K^+ channels are the largest known ion channel family and encompass more than 70 different genes of the mammalian genome (Gutman *et al.*, 2005; Steinlein, 2014). Each K^+ channel family member has a highly conserved amino acid sequence called the GYG (glycine-tyrosine-glycine) signature sequence, which helps form the K^+ selective pore in the channel (Steinlein, 2014). K^+ channels are the most widely distributed ion channels in living organisms and their functional diversity is further enhanced by alternative splicing, heteromeric assembly of different principal subunits, RNA editing and post-translational modifications (Coetzee *et al.*, 1999). Besides regulating the resting membrane potential, one of the key function of K^+ channels is to contribute to membrane repolarization and /or hyperpolarization of excitable cells (Steinlein, 2014).

K^+ channels have different structure and activation mechanisms. K^+ channels can be differentiated based on their structure for example if their subunits have eight, six, four or two transmembrane domains (Choe, 2002). K^+ channels can also be differentiated based on their activation mechanism such as if they are voltage- dependent (Yellen, 2002), voltage-independent (Cohen *et al.*, 2009), Ca^{2+}-activated (Faber *et al.*, 2003) or Na^+-activated (Bhattacharjee *et al.*, 2005a). K^+ channels are mostly known to exist as tetramers such that they are formed by co-assembly of four subunits that are specifically arranged in the cell membrane to form a central pore that allows K^+ movement through the middle of the complete channel assembly. The four subunits can either be same proteins or different proteins (belonging to another K^+ channel subfamily) in which case they are called homo-tetramers or hetero-tetramers, respectively (Choe, 2002; Yellen, 2002).

Although Na^+-activated K^+ channels, known as K_{Na} channels, have been known for about 30 years, they have not been as extensively characterized as the K_{Ca} channels. One possible reason

for this is because intracellular Na^+ is not as well-known as a second messenger as is intracellular Ca^{2+} (Bhattacharjee *et al.*, 2005a). However, the role of K_{Na} channels is now increasing being appreciated in neurons (Kim *et al.*, 2014) because intracellular Na^+ levels, like Ca^{2+} levels, have been found to increase significantly during electrical activity in neurons (Rose, 2002) and many neurons have been shown to express K_{Na} proteins (Kaczmarek, 2013).

The molecular identity of native K_{Na} channels is unknown but K^+ channels encoded by two genes known as Slack and Slick (both belonging to the slo 2 family) exhibit properties that are consistent with those expected of K_{Na} channels (Bhattacharjee *et al.*, 2005a; Kaczmarek, 2013). The slo family members derive their name from the *slowpoke* gene, which encodes the BK channel in *Drosophila* and mutation in which causes a characteristic lethargic and less-active phenotype. The term 'Slack' stands for ''*sequence like a calcium-activated potassium channel*'' which typically belong to slo 1 family encoding BK channels. The term 'Slick' on the other hand stands for ''*sequence like an intermediate conductance potassium channel*'' and get its name due to its structural similarity to slo 3 family that encodes IK channels (Wei *et al.*, 2005; Kaczmarek, 2013)]. Slack and Slick proteins both form tetrameric channels, which have six transmembrane domains (S1–S6) and form the pore between the S5 and S6 domains. Although there is about 70% homology between Slack and Slick, their biophysical properties are strikingly different. This has been attributed to differences in their cytoplasmic domains, especially the much shorter N terminal region and an additional ATP binding region that are present in Slick channels (Kaczmarek, 2013). For example, heterologously expressed Slick channels are known to rapidly activate upon step depolarizations and do not strictly depend on presence of intracellular Na^+ whereas Slack channels typically activate slowly but have an absolute dependence on Na^+. Slick channels are inhibited by the presence of intracellular ATP and are only activated when ATP level

decreases (as in hypoxia) while Slack channels are reportedly insensitive to intracellular ATP levels. Slick channels are also relatively more sensitive to presence of intracellular Cl^-, which is a positive modulator of both channels (Bhattacharjee *et al.*, 2005a). Although only one Slick isoform is known, alternate splicing of Slack RNA has been shown to produce five different isoforms of Slack in mice, which mostly differ in the structure of their N terminal regions (Brown *et al.*, 2008).

Although five different Slack isoforms are known to exist, the biophysical properties of only Slack A and Slack B have been studied in detail (Brown *et al.*, 2008). For example, Slack B was found to activate slowly while Slack A activates rapidly during step depolarizations. The unitary conductances of Slack B and Slack A are similar but the mean open time of Slack-B channels was over six times longer than that of Slack-A channels. Based on these biophysical properties and the use of numerical simulations, Slack-B are thought to promote bursting and a graded pattern of adaptation, whereas Slack-A channels are thought to contribute to a more consistent neuronal firing pattern (Brown *et al.*, 2008).

The original immunohistochemical report that demonstrated expression of Slack in rat brain actually used an antibody that was later found to target an epitope that is expressed only in the Slack B isoform (Bhattacharjee *et al.*, 2002; Brown *et al.*, 2008). Since the immunohistochemical pattern of Slick was found to overlap with that of Slack B in many regions, it suggested that native K_{Na} channels in those regions could be formed of heteromers of Slack B and Slick (Brown *et al.*, 2008). Indeed Slack B was later found to heteromerize with Slick and form channels that have properties that are different from those of either Slack B or Slick expressed alone (Chen *et al.*, 2009). Although Slack A was shown not to heteromerize with Slick (Chen *et al.*, 2009), the biophysical properties of other Slack isoforms and their possible heteromerization with

Slick and/or among themselves are still unknown. Finally, a detailed isoform-specific expression pattern of Slack has not been studied in brain, but it has been shown that there are brain regions that are enriched in specific isoforms (Brown *et al.*, 2008). Such an isoform-specific enrichment could be physiologically important to confer specific biophysical properties important for sustaining specific firing properties in those tissue. It will therefore be important to decipher the composition of native K_{Na} channels, which are thought to be formed of multimers of Slack and Slick subunits with unknown stoichiometries (Bhattacharjee *et al.*, 2005a).

1.6.1 Functions of K_{Na} channels

Since K_{Na} channels mediate outwardly rectifying K^+ conductance, their activation in principle would be expected to have a hyperpolarizing or inhibitory effect on the electrical activity of excitable cells. One of the first proposed physiological roles of K_{Na} channels was to confer cellular protection to cardiac myocytes under ischemic conditions (Kameyama *et al.*, 1984). It was thought that increases in intracellular Na^+ levels that would occur due to failure of Na^+/K^+-ATPase could activate K_{Na} channels in myocytes to keep their cellular excitability under control. However K_{Na} channels are present in many other cell types and their role is not limited to pathological conditions such as ischemia. For example, it has been shown that Na^+ influx following a single AP spike is sufficient to activate K_{Na} channels and to cause a fAHP in hippocampal and DRG neurons (Liu *et al.*, 2004; Gao *et al.*, 2008). The hyperpolarization mediated by K_{Na} channels has also been shown to summate and facilitate generation of the sAHP in lamprey spinal neurons (Wallén *et al.*, 2007), visual cortical neurons (Sanchez-Vives *et al.*, 2000), trigeminal neurons (Sandler *et al.*, 1998), thalamic neurons (Kim *et al.*, 1998) and neocortical intrinsically bursting neurons (Franceschetti *et al.*, 2003). The sAHP, as discussed before, is important in spike frequency adaptation and shaping of firing patterns in neurons, which suggests that K_{Na} channels are

important regulators of neuronal excitability. However, the time scale of activation of K_{Na} channels could depend on intracellular Na^+ dynamics in different neurons and on the subunits composition of K_{Na} channels. Although the major function of K_{Na} channels is to provide an inhibitory control during neuronal firing, studies in MNTB neurons have shown that K_{Na} channels could also play an important role in increasing the membrane conductance at resting potentials that is needed for accurate time matching of their electrical activity to the incoming high frequency auditory signals (Yang *et al.*, 2007). In electric fish, K_{Na} channels could take part in the repolarization process that follows the high frequency firing in the electrocytes that generates the high amplitude electric organ discharge needed during high activity periods of the fish. The presence of K_{Na} channels in their electric cells is believed to reduce the high energy demands that are otherwise created to sustain high-frequency and high amplitude APs (Markham *et al.*, 2013). In summary, K_{Na} channel are considered important for regulating the neuronal excitability by their AHP mechanisms and also facilitating neuronal adaptation to high frequency stimulations.

1.6.2 K_{Na} channels in MNCs

Although ion selective microelectrodes and ratiometric imaging techniques have shown that electrical activity (both single and multiple AP spikes) can cause an elevation of both the intracellular Ca^{2+} and Na^+ levels in neurons (Rose, 2002), only Ca^{2+} activated K^+ channels have been shown to contribute to generation of AHPs and to the regulation of firing properties of MNCs. A previous immunohistochemical report found a robust Slick expression (Bhattacharjee *et al.*, 2005b) but no expression of Slack B in the SON (Bhattacharjee *et al.*, 2002; Brown *et al.*, 2008). However, it remained possible that one or more other Slack isoforms could be present in the SON and form K_{Na} channels with or without Slick. I therefore hypothesized that rat MNCs express functional K_{Na} channels and therefore sought to test for their presence in acutely isolated

MNCs as discussed in Chapter 4 of this thesis.

1.6.3 Identifying functional K_{Na} channels

The study of macroscopic K_{Na} currents is considered difficult for two major reasons. First, their activation mechanisms are complex as they demonstrate both Na^+-dependent and voltage-dependent characteristics despite the fact that the typical voltage sensor present in all voltage dependent K^+ is not present in them (Kaczmarek, 2013). Second, there are no specific pharmacological inhibitors and activators for studying K_{Na} channels. Antiarrhythmic drugs like quinidine, bepridil and clofilium inhibit K_{Na} channels but they also inhibit many other ion channels belonging to Na^+, Ca^{2+} and K^+ channel families (de Los Angeles Tejada *et al.*, 2012). Similarly the K_{Na} channels activators like riluzole and niflumic acid also modulate Na^+ channels (Liu *et al.*, 1997), and many K^+ channels (Cheng *et al.*, 2009). The more common and simpler electrophysiology methods that are employed to test the presence of K_{Na} channels in native cell systems are therefore based on manipulation of intracellular Na^+ levels. For example; inhibition of Na^+ influx by Na^+ channel blocker tetrodotoxin or iso-osmotic substitution of external NaCl with LiCl (based on the fact that Li^+ permeates Na^+ channels but cannot activate the K_{Na} channels) have both been shown to cause inhibition of outwardly rectifying K^+ currents, thereby revealing the presence of K_{Na} channels. Similarly, loading of cells with solution containing high Na^+ through patch pipettes have been shown to cause an increase in the outward K^+ currents. The utility of these methods has been shown in more detail in chapter 4 of this thesis where I present evidence for the presence of functional K_{Na} channels in acutely isolated rat MNCs.

1.7 Rationale, hypothesis and objectives

MNCs are intrinsically osmosensitive and are key players in systemic osmoregulation. In response to changes in the osmotic conditions inside the body, they exhibit changes in their electrical activity and can regulate the systemic release of VP and OT, which are the two major osmoregulatory hormones. The studies performed in this thesis has the potential to help further understand the mechanisms that contribute to their intrinsic osmosensitivity and changes in electrical behaviour during osmotic changes.

1.7.1 Rationale for studying osmotically evoked PLC signaling mechanisms in MNCs

As was discussed in the introductory section of this thesis, osmotically-evoked changes in the mechanosensitive SICs is a key part of MNC osmosensitivity (Oliet *et al.*, 1993a; Oliet *et al.*, 1993b), which depends on osmotically-evoked structural changes in their intracellular cytoskeleton proteins (Zhang *et al.*, 2007b; Prager-Khoutorsky *et al.*, 2014). However it is unclear if MNCs also exhibit osmotically-evoked changes in their second messenger systems and if that could contribute to their osmosensitive properties.

As detailed in the chapter 2 and 3 of this thesis, I was interested in testing for osmotically-evoked changes in the PLC signalling system of MNCs for the following three reasons:

1) MNCs have been shown to express osmosensitive K_V7 and TRPV1 (Sharif Naeini *et al.*, 2006; Zhang *et al.*, 2009) channels, both of which activate under acute hypertonic conditions. Since both K_V7 and TRPV1 channels are widely known to have functional association with PIP_2 signalling mechanisms (Li *et al.*, 2005; Winks *et al.*, 2005; Brown *et al.*, 2007; Lukacs *et al.*, 2007; Rohacs, 2007; Ufret-Vincenty *et al.*, 2011), there was a possibility that PIP_2 pathways in MNCs might also exhibit osmosensitive changes and could therefore play a role in their osmotic physiology.

2) There have been reports that PLC activation may occur in response to stimuli that do not

involve GPCRs, such as application of TRPV1 channel agonists (Lukacs *et al.*, 2013; Borbiro *et al.*, 2015), mechanical stress (Ryan *et al.*, 2000), cold temperature (Daniels *et al.*, 2009), high K^+ depolarization (Kendall *et al.*, 1985; Lukacs *et al.*, 2013) and even osmotic treatment (Takahashi *et al.*, 2001; Komis *et al.*, 2008). Since acute hypertonic stimulation causes both mechanical stress and the opening of TRPV1 channels in the MNC membranes, there was a possibility that hypertonic stimuli could also cause PLC activation in MNCs.

3) Our laboratory had established an osmotically-evoked short term hypertrophy model in acutely isolated rat MNCs and showed that this is dependent on exocytotic fusion of intracellular membranes with the MNC membrane (Shah *et al.*, 2014). Since PKC activation by diacylglycerol (DAG) has been implicated in translocation of Ca^{2+} channels to the cell surface in molluscan neuroendocrine cells (Strong *et al.*, 1987) and of TRP channels in expressed cell systems (Morenilla-Palao *et al.*, 2004), there was possibility that a similar PLC hydrolysis-dependent mechanism might be involved in the osmotically-evoked hypertrophy response in MNCs.

1.7.2 Rationale for studying K_{Na} channels in MNCs

Ion selective microelectrodes and ratiometric imaging techniques have shown that electrical activity (both single and multiple AP spikes) can cause an elevation of both the intracellular Ca^{2+} and Na^+ levels in neurons (Rose, 2002). The intracellular increase in Na^+ and Ca^{2+} levels can activate K_{Na} and K_{Ca} channels, respectively. Both K_{Na} and K_{Ca} channels have been shown to contribute to the generation of AHPs in many different types of neurons and to shape their firing behaviour. Although K_{Ca} channels have been extensively studied and shown to contribute in generation of AHPs and regulation of firing properties of MNCs, the role of K_{Na} channels in MNCs has not been explored.

Although the molecular identity of K_{Na} channels is not known, the biophysical properties of

K_{Na} channels are consistent with those of expressed Slack and Slick proteins. The SON has a robust Slick expression (Bhattacharjee *et al.*, 2005b) but no expression of Slack B isoform (Bhattacharjee *et al.*, 2002; Brown *et al.*, 2008). However, since Slack has four more isoforms that were not tested in the SON, it remained possible that one or more of these Slack isoforms could be present in the SON and form K_{Na} channels with or without Slick. Based on these reports, I therefore hypothesized that rat MNCs express functional K_{Na} channels and therefore sought to test for their presence in acutely isolated MNCs as discussed in Chapter 4 of this thesis.

1.7.3 Hypotheses

There are two main hypotheses of this thesis;

1) Acute hypertonic stimulation causes rapid changes in the PIP_2 second messenger systems of MNCs and these osmotically-evoked PIP_2 changes are important determinants of the osmosensitivity of the MNCs.

2) MNCs express Slack and Slick proteins that form functional K_{Na} channels in the MNCs.

1.7.4 Major thesis objectives

1) Create an immunocytochemical protocol to allow me to study and quantify osmotically-evoked PIP_2 changes in the MNC plasma membrane (Chapter 2).

2) Characterize the mechanism of osmotically-induced PIP_2 changes in MNCs using the above model (Chapter 3).

3) Study the impact of osmotically-induced PIP_2 changes on the function of TRPV1-encoded SICs in MNCs using whole cell patch clamp methods (Chapter 3).

4) Test for Slack (slo 2.2) and Slick (slo 2.1) proteins in MNCs using immunostaining methods (Chapter 4).

5) Test for functional K_{Na} channels in MNCs (Chapter 4).

Preface to Chapter 2

The following chapter is a published paper that was published in Journal of Physiology in year 2014. The full citation of this paper is as follows;

Osmotic activation of phospholipase C triggers structural adaptation in osmosensitive rat supraoptic neurons. J Physiol. 2014 Oct 1; 592(19):4165-75.

Shah L*****, **Bansal V***, Rye PL, Mumtaz N, Taherian A, Fisher TE

*** denotes equal first authors**

Being the co-first author of this paper, I have made few minor modifications like changing the page numbers, figure numbers and rewording of a few sentences in the original manuscript to make it fit in my manuscript based thesis format.

The paper had in total 5 figures and 3 different techniques were used to generate data for this paper which includes morphometry (cell surface measurements), whole cell patch clamp (membrane capacitance) and immunocytochemistry (membrane PIP_2 estimations using PIP_2 antibody). My experimental contributions in this paper includes exclusively doing all PIP_2 immunocytochemistry and whole cell patch clamp experiments on acutely isolated rat MNCs. I am therefore responsible for all the data that was shown in Figure 3 and 4 of this paper. Parts of this paper (Figure 1, 2 and 5 and data related to these figures) have been included in the Master's theses of former students Ms. Naima Mumtaz and Mr. Love Shah and who have graduated from Dr Fisher's lab in years 2011 and 2014 respectively.

In this paper, I have reported the novel evidence that demonstrated the presence of an osmotically-evoked intracellular PLC signalling pathway in rat MNCs in Figure 4. Specifically I have shown that an acute 5 minute hypertonic saline exposure of acutely isolated rat MNCs can cause

a significant decrease in their membrane PIP_2 levels in a PLC dependent fashion. I have also made a population comparison of the cell membrane capacitance values of acutely isolated rat MNCs that were either exposed to a short term (90 minutes) hypertonic exposure or were maintained in isotonic saline during this time using whole cell patch clamp methods (Figure 3). My electrophysiology data suggested that the cells exposed to short term hypertonic conditions had a significantly higher averaged capacitance values in comparison to the control cells that were maintained in isotonic conditions which lended support to the short term hypertrophy in rat MNCs that we reported in this paper.

CHAPTER 2

OSMOTIC ACTIVATION OF PHOSPHOLIPASE C TRIGGERS STRUCTURAL ADAPTATION IN OSMOSENSITIVE RAT SUPRAOPTIC NEURONS

2.1 ABSTRACT

The magnocellular neurosecretory cells of the hypothalamus (MNCs) synthesize and secrete vasopressin or oxytocin. A stretch-inactivated cation current mediated by TRPV1 channels rapidly transduces increases in external osmolality into a depolarization of the MNCs leading to an increase in AP firing and thus hormone release. Prolonged increases in external osmolality, however, trigger a reversible structural and functional adaptation that may enable the MNCs to sustain high levels of hormone release. One poorly understood aspect of this adaptation is somatic hypertrophy. We demonstrate that hypertrophy can be evoked in acutely isolated rat MNCs by exposure to hypertonic solutions lasting tens of minutes. Osmotically-evoked hypertrophy requires activation of the stretch-inactivated cation channel, AP firing, and the influx of Ca^{2+}. Hypertrophy is prevented by pretreatment with a cell-permeant inhibitor of SNARE-dependent fusion and is associated with an increase in total membrane capacitance. Recovery is disrupted by an inhibitor of dynamin function, suggesting that it requires endocytosis. I have demonstrated that hypertonic solutions cause a decrease in phosphatidylinositol 4, 5-bisphosphate (PIP_2) in the plasma membranes of MNCs that is prevented by an inhibitor of phospholipase C. Inhibitors of phospholipase C or protein kinase C prevent osmotically-evoked hypertrophy and treatment with a protein kinase C-activating phorbol ester can elicit hypertrophy in the absence of changes in osmolality. These studies suggest that increases in osmolality cause fusion of internal membranes with the plasma membrane of the MNCs and that this process is mediated by activity-dependent activation of phospholipase C and protein kinase C.

2.2 INTRODUCTION

The magnocellular neurosecretory cells (MNCs) of the supraoptic and paraventricular nuclei of the hypothalamus are osmosensitive (Bourque, 2008) in that their electrical excitability is altered by changes in external osmolality. These cells regulate body fluid balance by releasing more vasopressin (VP) and oxytocin (OT) as extracellular osmolality increases (Bourque, 2008). MNCs show a decrease in volume and thus plasma membrane tension in response to acute increases in osmolality and lack the acute compensatory mechanisms (Zhang *et al.*, 2003b) that limit osmotically-evoked volume changes in most cell types (Hoffmann *et al.*, 2009). This enables MNCs to faithfully transduce increases in osmolality into a depolarizing current through activation of a stretch-inactivated cation channel (SIC; (Oliet *et al.*, 1993a) mediated by a variant of the TRPV1 (transient receptor potential cation channel vanilloid subfamily member 1) channel (Sharif Naeini *et al.*, 2006). Increases in osmolality lasting hours, however, also activate a broad and dramatic functional and structural transformation of the MNCs that is thought to enable sustained high levels of hormone release. Similar changes occur in OT-releasing MNCs during lactation and parturition, when the release of OT is elevated (Theodosis *et al.*, 2008). Osmotically-evoked adaptations include structural changes such as retraction of the glial processes surrounding the MNCs (Theodosis *et al.*, 2008) and increased synaptic innervation (Tasker *et al.*, 2002), and also functional changes such as up-regulation of many genes (Ghorbel *et al.*, 2003), and increases in the cell surface expression of many channels and receptors (Shuster *et al.*, 1999; Tanaka *et al.*, 1999; Hurbin *et al.*, 2002; Zhang *et al.*, 2007a). One aspect of this adaptation is a marked and reversible hypertrophy of the MNC somata (Miyata *et al.*, 2002).

The sequence of events that triggers and mediates MNC hypertrophy is unknown and

difficult to elucidate using *in vivo* models. We therefore sought to test whether exposure to hypertonic saline could evoke hypertrophy in MNCs acutely isolated from adult rats. We observed that MNCs undergo hypertrophy when exposed to increases in external osmolality lasting tens of minutes and that this occurs in response to increases in external osmolality that would be expected to occur physiologically. We present evidence that the initiation and maintenance of osmotically-induced hypertrophy is activity-dependent and occurs through SNARE-dependent exocytotic fusion of internal membranes with the MNC plasma membrane. Furthermore, I have shown that exposure of isolated MNCs to hypertonic solutions causes a rapid increase in the activity of the enzyme phospholipase C and that this activation appears to be central to the initiation of osmotically-evoked hypertrophy. Our results demonstrate a mechanism that is likely to underlie at least part of the osmotically-induced hypertrophy that has been observed in mammalian MNCs *in situ* and suggests that MNC somata may undergo dynamic structural regulation *in vivo* in response to changes in external osmolality within the physiological range.

2.3 METHODS

2.3.1 Ethical approval

This work was approved by the University of Saskatchewan's Animal Research Ethics Board, and adhered to the Canadian Council on Animal Care guidelines for humane animal use.

2.3.2 Animals and Cell Preparation

MNCs were isolated using a protocol described previously (Liu *et al.*, 2005) and identified using the criterion established by Oliet and Bourque (Oliet *et al.*, 1992), i.e. a maximal cross sectional

area (CSA) greater than 160 μm^2. In brief, male Long-Evans rats (200-300 g) were anaesthetized with halothane and killed by decapitation. The brain was removed and blocks of tissue containing most of the two supraoptic nuclei were excised. The tissue blocks were incubated with an oxygenated (100% O_2) PIPES solution (pH 7.1) composed of (in mM): NaCl, 110; KCl, 5; $MgCl_2$, 1; $CaCl_2$, 1; PIPES, 20; glucose, 25; containing trypsin (Type XI, 0.6 mg/ml) for 90 min at 34^o C. After incubation, the tissues were then transferred into oxygenated PIPES solution without trypsin for 30 minutes at room temperature. Finally, the tissues were gently triturated with fire-polished pipettes to disperse the cells, which were plated onto glass bottomed culture dishes and kept at room temperature for use the same day. Hippocampal neurons were isolated from hippocampal tissue blocks obtained from adult rats using a similar protocol. The osmolalities of the external solutions were adjusted before each experiment to 295 ± 3 mosmol kg^{-1} or as noted in the text using a VAPRO pressure osmometer (WESCOR) by adding mannitol as required.

2.3.3 Hypertrophy experiments

In some experiments, the MNCs were perfused with oxygenated isotonic PIPES saline, switched to hypertonic saline at the indicated osmolality, and then returned to isotonic saline. In other experiments, MNCs were exposed to stationary bath solutions of defined osmolality, with or without the addition of chemicals, as indicated in the text. Healthy-looking MNCs (typically 2-3 per dish) were photographed at the indicated times with a cooled CCD camera attached to a Zeiss Axiovert 200 using a 40X objective. The maximal circumference of the cell soma was traced and the CSA determined using ImageJ (NIH). MNCs that failed to shrink in response to application of hypertonic solution or to recover toward baseline when they were returned to isotonic solution were considered unhealthy and were excluded from further analysis. Following rapid shrinkage,

most MNCs showed a slow hypertrophy to at least their baseline CSA in both the perfusion studies shown in Figure 2.1B (12 out of 15 MNCs treated with 325 mosmol kg^{-1} and 10 out of 12 MNCs treated with 305 mosmol kg^{-1}), Figure 2.1C (in the presence of bumetanide; 10 out of 12), and Figure 2.2D (10 out of 13), and for the stationary bath experiments shown in Figure 2.1D (17 out of 21 MNCs), Figure 2.2B (21 out of 24), and Figure 2.2C (in the presence of the scrambled version of the TAT-NSF700scr peptide; 19 out of 19). We do not know if the MNCs that do not hypertrophy are a distinct subset of MNCs or have incurred some form of damage during the isolation procedure that prevents them from being activated by hypertonic saline or from undergoing hypertrophy. We did not include data on MNCs that did not hypertrophy in the plots shown to give a better indication of the hypertrophic response. Inclusion of the MNCs that did not undergo hypertrophy in response to hypertonic treatment does not change the level of significance of any of the statistical comparisons shown in the results section. Data were normalized by dividing by each measurement by the mean CSA of that cell during the control period and are expressed as mean ± SEM. For the fluorescent images shown in Figure 2.1A, MNCs were incubated with the membrane dye CellMask Orange (Invitrogen; 5 μg/ml) for 5 minutes and then rinsed with isotonic saline three times. Fluorescent imaging was performed as described below.

2.3.4 Electrophysiological methods:

The plasma membrane capacitances of acutely isolated rat MNCs were determined using whole cell patch clamp at room temperature. The values for MNCs exposed to hypertonic (325 mosmol kg^{-1}) saline for 90 minutes or more were compared to these of MNCs maintained in isotonic (295 mosmol kg^{-1}) saline. Borosilicate glass capillaries (1.2 mm O.D, 0.68 I.D; A-M Systems) were used

to pull patch pipettes on a P-97 horizontal pipette puller (Sutter Instrument Company) and fire-polished using a microforge (Narashige). They were filled with an internal solution containing (in millimolar) 140 KCl, 10 HEPES, 1 $MgCl_2$, 1 EGTA, and 1 Mg-ATP (pH 7.2) and had a resistance of 2-4 MΩ. The whole cell membrane capacitances of MNCs were estimated using an EPC-9 amplifier (HEKA) controlled with PULSE software (HEKA), using the auto C-Slow function of PULSE. Data are expressed as mean ± SEM.

2.3.5 Immunocytochemistry:

Acutely isolated rat MNCs were incubated in PIPES saline with or without the PLC inhibitor U73122 (Enzo Life Sciences) for 20 minutes and then stimulated with either hypertonic PIPES saline (325 mosmol kg^{-1}) or isotonic saline containing 10 µM oxotremorine (Sigma) for 5 minutes. The control cells were left untreated. The cells were then subjected to PIP_2 immunostaining in rat MNCs using a modification of a published protocol (Hammond *et al.*, 2006). Briefly, the cells were fixed with phosphate-buffered saline (PBS) containing 4% paraformaldehyde and 0.1% glutaraldehyde for 20-25 minutes at room temperature. Following 3 washes with PBS, the cells were blocked with solution containing 10% donkey serum and 0.5% saponin for 1 hour. The cells were then incubated with a mouse monoclonal PIP_2 antibody (Enzo Life Sciences; 1:1000) overnight at 4^0C. The dishes were washed with PBS three times and incubated for another 1 hour with donkey anti-mouse secondary antibody (Invitrogen Alexa Fluor 488, 1; 1000). After three washes with PBS, Citifluor mounting solution (Citifluor) was added to the dishes and cells were then viewed using a Zeiss inverted Axiovert- 200 microscope with appropriate filter sets and a 40X objective and images were captured using a cooled CCD camera. The images were analyzed using ImageJ software (NIH) by tracing the perimeter of each soma by following the line of greatest

fluorescence (disregarding processes) and determining the mean fluorescence of pixels on that line. The mean intensities of staining for all MNCs in each treatment group were normalized to the mean fluorescence of all of the control cells on each experimental day. Data are expressed as normalized mean ± SEM.

2.3.6 Chemicals

All chemicals, unless stated otherwise, were from Sigma-Aldrich Corporation. The TAT-NSF700 peptide and its scrambled version were purchased from AnaSpec, Inc. and were used at a concentration of 1.2 μM.

2.4 RESULTS

We sought to determine if an increase in osmolality can trigger hypertrophy in MNCs acutely isolated from adult rats and, if so, to elucidate the underlying mechanisms. We used the maximal cross sectional area (CSA) of the MNCs to monitor changes in volume, as has been used previously (Zhang *et al.*, 2003b), and observed that treatment with hypertonic saline caused rapid cell shrinkage followed by slower cell enlargement. This is illustrated in Figure 2.1A, which shows an acutely isolated MNC and the shrinkage and enlargement of that cell following treatment with hypertonic saline. Note that the fluorescent membrane dye used to obtain these images was for demonstration purposes only; in all of the other experiments we measured the cell perimeter using differential interference contrast (DIC) images of the MNCs to monitor their CSA. To determine the time course of these changes, MNCs (n = 12) were perfused with an oxygenated saline solution with an osmolality close to the normal set point in the rat (i.e. isotonic or 295 mosmol kg^{-1}) and then switched to a hypertonic saline (325 mosmol kg^{-1}). MNCs rapidly shrunk to approximately 94% of control (a reduction of mean CSA from 363 ± 36 μm^2 to 343 ± 36 μm^2;

Figure 2.1B), but after a delay of about 20 minutes started to hypertrophy and achieved a peak size of approximately 105% of control (381 ± 38 μm^2) after about 1 hour (Figure 2.1B). The mean CSA during the shrunken and enlarged states (measured 5 and 75 minutes after the beginning of perfusion of hypertonic saline, respectively) were both significantly different than the mean baseline CSA (using a one-way repeated measures analysis of variance test; $P < 0.01$ in both cases). Smaller amounts of shrinkage and hypertrophy were observed (Figure 2.1B) when MNCs were perfused with 305 mosmol kg^{-1} saline (98% and 103%; n=10), but these differences were also significant (using a one-way repeated measures analysis of variance test; $P < 0.01$ in both cases). MNCs rapidly recovered to their control size when returned to isotonic saline and no changes in size were observed in MNCs maintained for similar time periods in isotonic saline. The mean CSA during the shrunken and enlarged states following perfusion with 325 mosmol kg^{-1} or 305 mosmol kg^{-1} saline were also significantly different than the mean CSA of MNCs perfused with isotonic saline for similar periods (using a two-way analysis of variance; $P < 0.01$ in all cases). The hypertrophic response did not appear to be altered by inhibition of the Na^+-K^+-Cl^- cotransporter NKCC1, which is commonly involved in cell volume regulation, by the antagonist bumetanide (10 µM; Figure 2.1C). Experiments that were conducted using a stationary bath showed a similar pattern of hypertrophy in response to hypertonic saline (Figure 2.1D), but acutely isolated hippocampal neurons did not display osmotically-evoked hypertrophy (Figure 2.1D), suggesting that the response is specific to the MNCs.

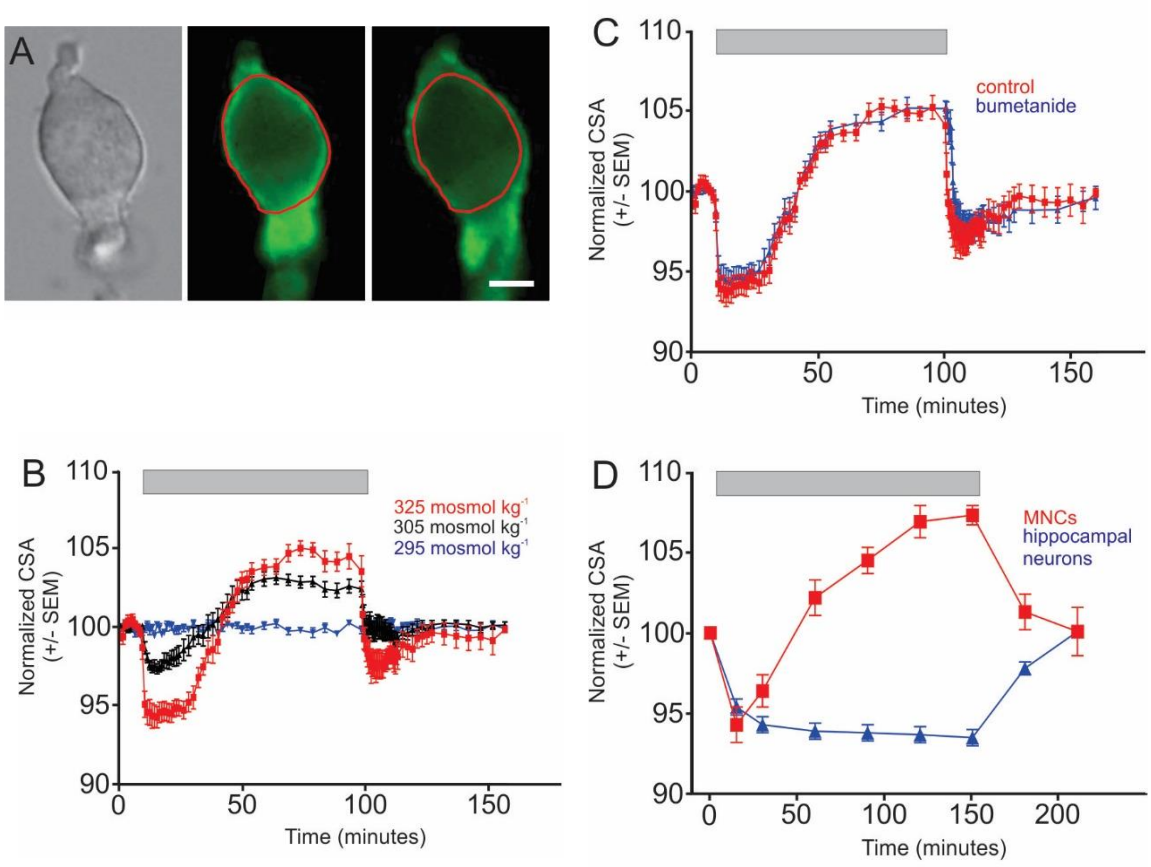

Figure 2.1. Increases in osmolality evoke reversible hypertrophy in osmosensitive supraoptic neurons but not hippocampal neurons. A, Images of an acutely isolated MNC showing osmotically-evoked cell shrinkage and hypertrophy. The image on the left shows a DIC image of an isolated MNC in isotonic saline. The two images to the right show the fluorescence of a plasma membrane dye (CellMask Orange; see Methods) in the same cell 5 and 80 minutes after administration of hypertonic saline. The red line shows the perimeter of the cell under isotonic conditions for comparison. Note that the cell in the center image shows shrinkage relative to the red line and the right image shows enlargement relative to the red line. The scale bar indicates 10 µm. B, Perfusion of oxygenated hypertonic saline (325 and 305 mosmol kg^{-1}) causes isolated MNCs to shrink and then hypertrophy over tens of minutes (n = 12 and 10, respectively) whereas perfusion with isotonic saline (295 mosmol kg^{-1}) has no effect. The period of perfusion is indicated by the bar at the top of the plot. Return to isotonic saline causes the cells to return to their original size. C, The response to perfusion of hypertonic saline (325 mosmol kg^{-1}) was not affected by the presence of bumetanide (10 µM; n = 10), which is an inhibitor of the Na^+-K^+-Cl^- co-transporter NKCC1. The response of the MNCs to perfusion of hypertonic saline (325 mosmol kg^{-1}) is shown for comparison (and is labeled "control"). D, Similar results were seen with MNCs that were maintained in a stationary bath that was switched to a hypertonic saline (325 mosmol kg^{-1}) and then back to isotonic saline (n = 17). Isolated hippocampal neurons respond with shrinkage, but not hypertrophy, to this treatment (n = 20).

Preincubation with the Na^+ channel blocker tetrodotoxin (TTX; 0.2 µM) prevented hypertrophy (Figure 2.2A), demonstrating that the response is dependent upon the activation of electrical activity. Hypertrophy was also prevented by the SB366791 (1.5 µM), which blocks TRPV1 channels (and more specifically the SIC; (Sharif-Naeini *et al.*, 2008), suggesting that activation of the SIC is necessary for hypertrophy, by the cell permeant Ca^{2+} chelator BAPTA-AM (10 µM), suggesting that an increase in intracellular Ca^{2+} is required, and by the L-type Ca^{2+} channel blocker nifedipine (10 µM), suggesting that the effect depends on Ca^{2+} influx through L-type Ca^{2+} channels (Figure 2.2A). These data suggest that increases in external osmolality cause MNC shrinkage, leading to the activation of the SIC, an increase in the firing of APs, and an increase in Ca^{2+} influx through L-type Ca^{2+} channels, and that the resultant increase in intracellular Ca^{2+} somehow activates hypertrophy. The addition of TTX, SB366791, or nifedipine to MNCs in hypertonic solutions following a hypertrophic response caused its reversal (Figure 2.2B), suggesting that the maintenance of hypertrophy is dependent on continued electrical activity and Ca^{2+} influx and that the cessation of Ca^{2+} influx leads to the reversal of the process. These data also suggest that MNCs continue to fire actions potentials even when their surface area has been significantly enlarged and that hypertrophy does not therefore decrease activity of the SIC.

We attempted to block the hypertrophic response using TAT-NSF700 (Matsushita *et al.*, 2005), a peptide that prevents SNARE-mediated exocytotic fusion by blocking the function of NSF (*N*-ethylmaleimide-sensitive factor). Although the presence of a scrambled version of the peptide had no apparent effect on the response of the MNCs to increased osmolality, hypertrophy was virtually eliminated by preincubation with TAT-NSF700 (n = 57; Figure 2.2C), suggesting that hypertrophy depends on SNARE-mediated exocytotic fusion. The mean CSA of hypertrophied MNCs incubated with 325 mosmol kg^{-1} saline in the presence of the scrambled peptide was

significantly larger than the mean CSA of MNCs incubated with 325 mosmol kg^{-1} saline in the presence of TAT-NSF700 (using a two-way analysis of variance; $P < 0.01$). Dynasore (80 µM), an inhibitor of dynamin-dependent endocytosis, was applied to MNCs in hypertonic saline (325 mosmol kg^{-1}) to test whether the rapid recovery of MNC cell size following hypertrophy requires membrane internalization. Dynasore prevented the recovery of MNCs to their original size when they were returned to isotonic saline, suggesting that the recovery process involves endocytotic retrieval of membrane from the MNC plasma membrane (Figure 2.2D).

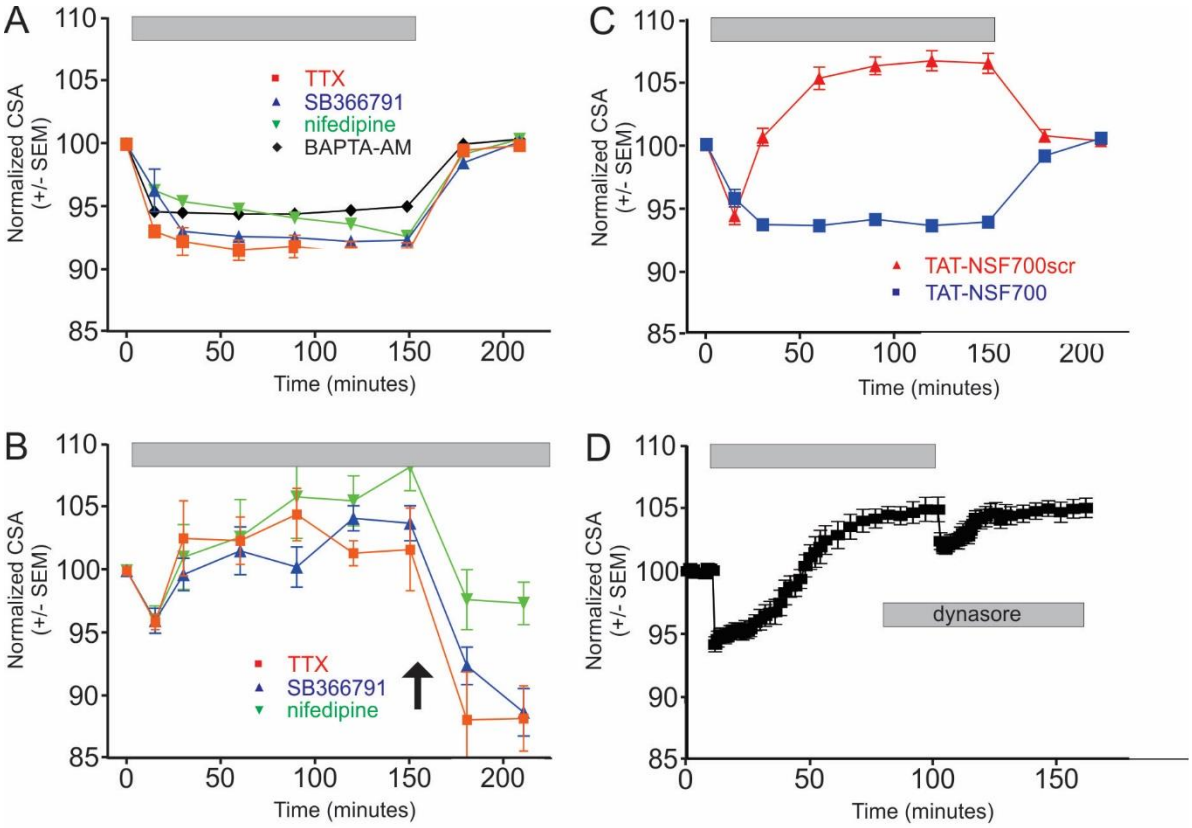

Figure 2.2. The initiation and maintenance of osmotically-evoked hypertrophy depends upon cell firing and Ca^{2+} influx and involves exocytotic fusion. A, Hypertrophy is prevented by treatment with tetrodotoxin (0.2 µM; n = 24), SB336791 (1.5 µM; n = 26), nifedipine (10 µM; n = 27), or BAPTA-AM (10 µM; n = 20). B, Hypertrophy is reversed in hypertonic saline by application (at arrow) of TTX (0.2 µM; n = 6), SB355791 (1.5 µM; n = 7), or nifedipine (10 µM; n = 7). C, Hypertrophy is prevented by administration of the cell permeant peptide TAT-NSF700 (n = 57), which blocks SNARE-mediated exocytotic fusion, but not the scrambled version of the peptide (TAT-NSF700scr; n = 19). D, The administration of dynasore (80 µM), an inhibitor of dynamin-mediated endocytosis, inhibits recovery from osmotically-evoked hypertrophy (n = 10).

I tested whether osmotically-evoked hypertrophy was associated with an increase in plasma membrane area by measuring the cell capacitance of isolated MNCs using whole cell patch clamp techniques. I found (Figure 2.3) that the whole cell capacitance was larger in MNCs that had been exposed to hypertonic (325 mosmol kg^{-1}) solutions for at least 90 minutes (16.7 ± 0.4 pF; n = 71) compared to that of MNCs maintained in isotonic (295 mosmol kg^{-1}) solution (15.6 ± 0.3 pF; n = 66; $p < 0.05$). These data support the hypothesis that the hypertrophic response involves the fusion of internal membranes with the MNC plasma membrane.

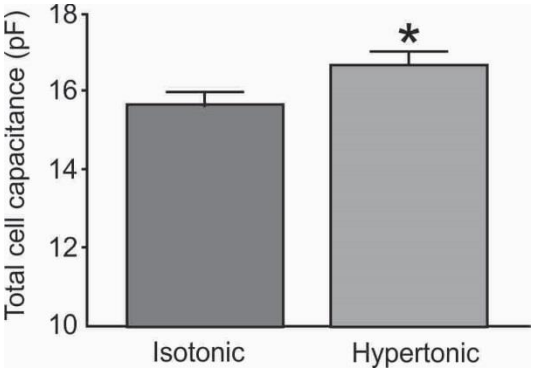

Figure 2.3. Exposure to hypertonic saline causes an increase in the total plasma membrane capacitance of isolated MNCs. The bars indicate the mean capacitance measured using whole cell patch clamp in isolated cells maintained in isotonic (295 mosmol kg^{-1}) or hypertonic (325 mosmol kg^{-1}) saline for at least 90 minutes. MNCs exposed to hypertonic saline had a greater total membrane capacitance (16.7 ± 0.4 pF; n = 71) than MNCs maintained in isotonic saline (15.6 ± 0.3 pF; n = 66). Data is expressed as mean ± SEM ($P < 0.05$).

Activation of PKC by DAG has been implicated in translocation to the cell surface of Ca^{2+} channels in molluscan neuroendocrine cells (Strong *et al.*, 1987) and of TRP channels in neurons (Morenilla-Palao *et al.*, 2004) and we therefore sought to determine whether such a mechanism could be involved in osmotically-evoked fusion of internal membranes with the MNC plasma membrane. DAG is produced by the cleavage of PIP_2 by the enzyme PLC and I therefore tested whether exposure to high osmolality causes a decrease in PIP_2 immunoreactivity in isolated MNCs. I found robust PIP_2 immunoreactivity in the plasma membrane of acutely isolated MNCs and that this immunoreactivity was reduced by exposure to hypertonic saline (Figure 2.4A and 2.4B). When the data from all cells are normalized to the mean intensity of staining in control cells I found that the level of staining in MNCs treated for five minutes with hypertonic saline (72.5 ± 3.4; n = 254 cells in 7 experiments) was decreased compared to that in control cells (100 ± 3.8; n = 276 cells in 7 experiments; $P < 0.01$ using a paired t test), and that this difference was prevented by pretreatment with the PLC inhibitor U73122 (104.7 ± 2.8; n = 303 cells in 7 experiments). These data suggest that exposure to hypertonic saline causes a decrease in membrane PIP_2 levels through the activation of PLC. Treatment of MNCs with the muscarinic receptor agonist oxotremorine also causes a decrease in PIP_2 immunoreactivity (68 ± 4.3; n = 155 cells in 4 experiments; $P < 0.05$ using a paired t test) that is prevented by U73122 (97.7 ± 3.9; n = 127 cells in 4 experiments).

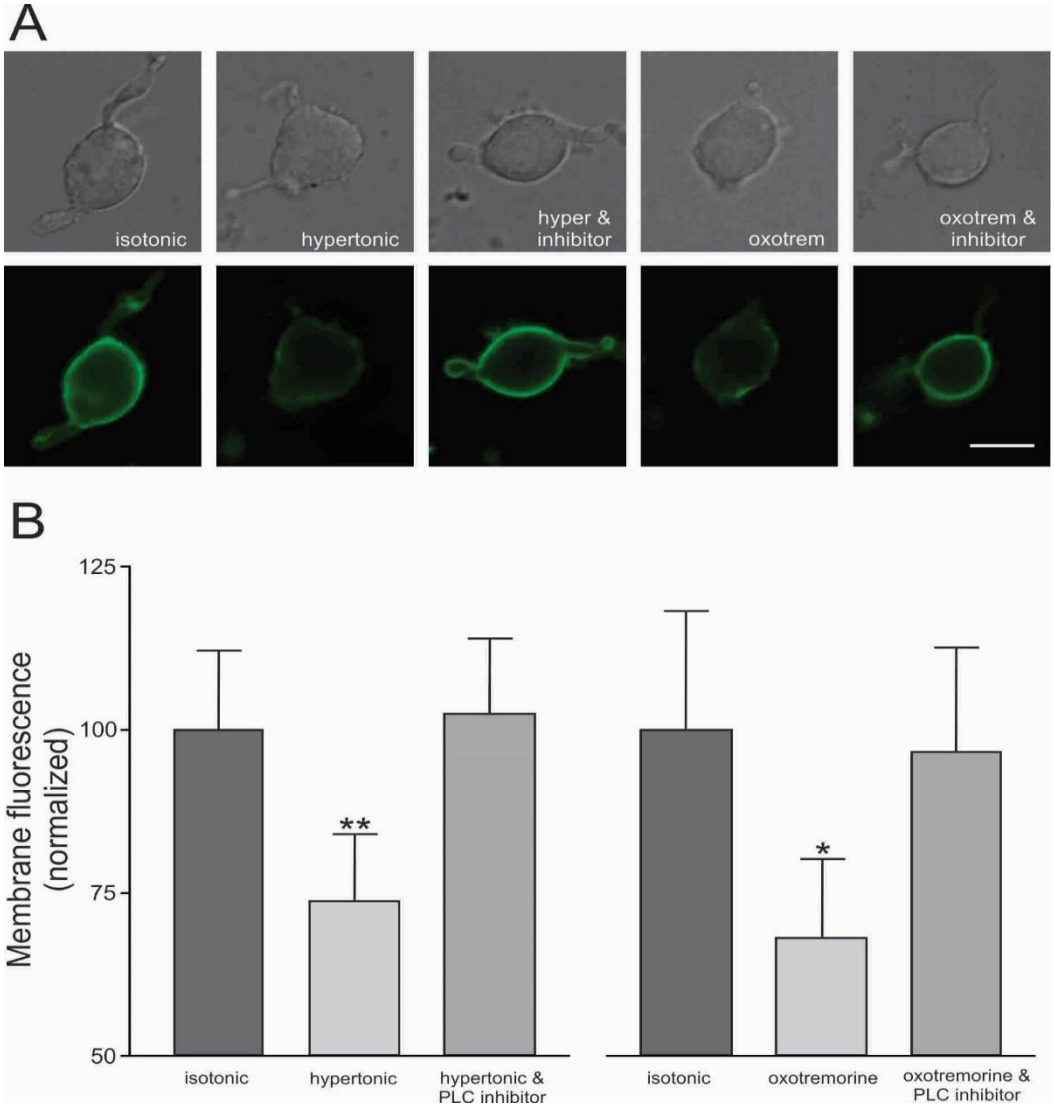

Figure 2.4. Exposure to hypertonic saline causes a decrease in immunoreactivity to PIP_2 in the plasma membrane of isolated MNCs. A, Images of isolated MNCs using either differential interference contrast images (upper panels) or fluorescence images showing immunoreactivity for PIP_2 (lower panels). MNCs were maintained in isotonic saline (control), or exposed to hypertonic saline (hypertonic), hypertonic saline with the PLC inhibitor U73122 (hyper & inhibitor), the muscarinic agonist oxotremorine (oxotrem), or oxotremorine and U73122 (oxotrem & inhibitor). B, The bar graph to the left shows the normalized immunoreactivity to PIP_2 in MNCs maintained in isotonic saline (control; 100.0 ± 12.0; n = 276 cells in 7 experiments) exposed to hypertonic saline (73.7 ± 10.5; n = 254 cells in 7 experiments), and hypertonic saline with the PLC inhibitor U73122 (102.4 ± 11.6; n = 303 cells in 7 experiments). The bar graph on the right shows the normalized immunoreactivity to PIP_2 in MNCs maintained in isotonic saline (control; 100.0 ± 18.2; n = 139 cells in 4 experiments), exposed to the muscarinic agonist oxotremorine (68.1 ± 12.1; n = 155 cells in 4 experiments), and exposed to oxotremorine and U73122 (96.6 ± 16.0; n = 127 cells in 4 experiments). Data are expressed as mean normalized fluorescence intensity ± SEM.

We then exposed MNCs to hypertonic solutions in the presence of inhibitors of PLC and PKC to test whether the activation of PLC is required for osmotically-evoked hypertrophy. MNCs exposed to hypertonic saline (325 mosmol kg^{-1}) in the presence of either a PLC inhibitor (U73122; 1 μM) or a PKC inhibitor (bisindolylmaleimide I; 1 μM) displayed osmotically-induced cell shrinkage but not hypertrophy (Figure 2.5A). The mean CSA of MNCs at the end of the incubation with 325 mosmol kg^{-1} saline in the presence of either of the two inhibitors was significantly smaller than the mean CSA of MNCs incubated in their absence (using a two-way analysis of variance; $P < 0.01$ in both cases). Furthermore, application of the PKC activator phorbol 12-myristate 13-acetate (0.1 μM) induced hypertrophy in the absence of an increase in osmolality in 7 out of 10 cells tested. The mean response of the cells that showed enlargement is shown in Figure 2.5A. The inactive phorbol ester 4α-phorbol 12-myristate 13-acetate (0.1 μM) caused no change in cell size (not shown). The mean CSA of MNCs treated with the PKC activator was significantly larger than the mean CSA of MNCs treated with the inactive phorbol analog (using a two-way analysis of variance; $P < 0.01$). Hypertrophy was also evoked by addition of the Ca^{2+} ionophore A23187 (10 μM) in isotonic solution or by exposure to isotonic saline with an elevated (25 mM) concentration of K^+ (Figure 2.5B), which would be expected to depolarize the resting membrane potential of the MNCs to about -40 mV. This depolarization could result in Ca^{2+} influx by triggering the firing of APs or it could cause influx of Ca^{2+} through the low-voltage activated L-type Ca^{2+} channels that are expressed in MNCs (Fisher *et al.*, 1995). Hypertrophy evoked by high K^+ concentrations was also prevented by the presence of U73122 (1 μM; Figure 2.5B). The mean CSA of MNCs incubated with high K^+ saline was significantly larger than the mean CSA of MNCs incubated with high K^+ saline in the presence of the PLC inhibitor (using a two-way analysis of variance; $P < 0.01$). These results are consistent with the hypothesis that osmotically-evoked

hypertrophy depends upon activity-dependent Ca^{2+} influx leading to the activation of PLC and, through an increase in the concentration of DAG, activation of PKC.

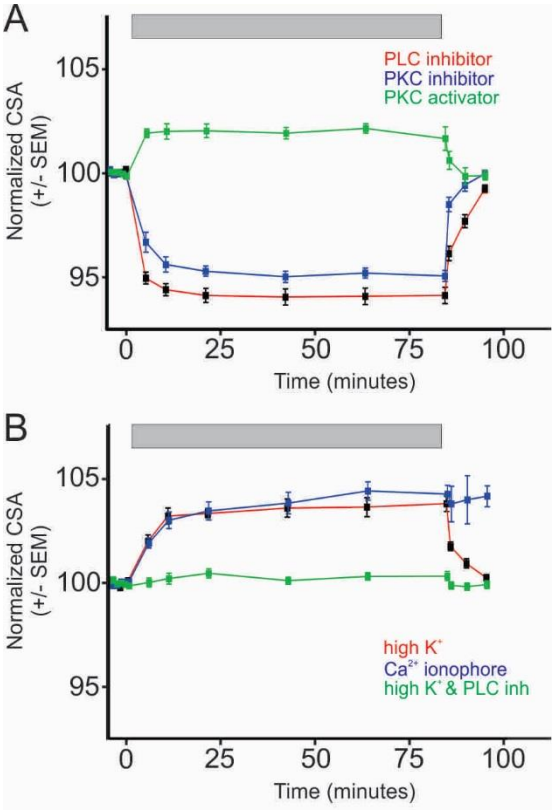

Figure 2.5. Osmotically-evoked hypertrophy is prevented by inhibitors of PLC or PKC and hypertrophy may be activated by a Ca^{2+} ionophore or by exposure to high K^+ saline. A, Exposure (indicated by grey bar) to hypertonic (325 mosmol kg^{-1}) saline causes MNC shrinkage, but not hypertrophy, in the presence of the PLC inhibitor U73122 (1 µM) or the PKC inhibitor bisindolylmaleimide I (1 µM). Exposure to the PKC activator phorbol 12-myristate 13-acetate (0.1 µM; green symbols and line) induced hypertrophy in the absence of an increase in osmolality. **B,** Exposure (indicated by grey bar) to either high K^+ saline (25 mM) or to the Ca^{2+} ionophore A23187 (10 µM) causes MNCs to hypertrophy. The hypertrophic effect of high K^+ saline is blocked by the PKC inhibitor bisindolylmaleimide I (1 µM).

2.5 DISCUSSION

The MNCs and the astrocytes that surround them undergo a remarkable structural and functional transformation in response to sustained increases in external osmolality. The astrocytes in both the hypothalamus and the neurohypophysis retract their processes from around the MNCs (Theodosis *et al.*, 2008) and the latter astrocytes also show an up-regulation of L-type Ca^{2+} channels (Wang *et al.*, 2009). In addition to the hypertrophy of the MNC somata, MNCs show increased synaptic innervation (Tasker *et al.*, 2002), up-regulation of the genes coding for VP (Zingg *et al.*, 1986) and other peptides (Ghorbel *et al.*, 2003; Hindmarch *et al.*, 2006), an increase in L-type Ca^{2+} current in MNC somata (Zhang *et al.*, 2007a), an increase in the expression of the V_{1a} vasopressin receptor on the MNC membrane (Hurbin *et al.*, 2002), translocation of the dynorphin receptor to the MNC terminal plasma membrane (Shuster *et al.*, 1999), and an increase in the amplitude of transient and persistent Na^+ currents (Tanaka *et al.*, 1999). The somatic hypertrophy that forms the focus of this work was first identified using electron microscopy to observe the increase in CSA of MNC somata in rats that had been dehydrated. MNCs have been reported to actually double in size (Armstrong *et al.*, 1977). This increase appears to involve an increase in total membrane area as MNCs isolated from dehydrated rats have been shown to have membrane capacitance values that are 20 to 33% larger than those from normally hydrated rats (Tanaka *et al.*, 1999; Di *et al.*, 2004; Zhang *et al.*, 2007a). The time course of hypertrophy may be quite rapid; changes in cell size have been detected in rat MNCs following water deprivation of as little as 2 hours (Hatton *et al.*, 1973) and injection of hypertonic saline can cause some structural changes in 30 minutes (Tweedle *et al.*, 1993). The mechanisms underlying hypertrophy and its physiological consequences are poorly understood, in part because hypertrophy is difficult to study using *in vivo* models.

Our data suggest that some portion of the hypertrophic response can be observed in acutely isolated MNCs and that no other cells are required to initiate this process. The fact that these changes can be evoked by changes in the concentration of mannitol in the bathing solution suggests that an increase in external osmolality is a sufficient trigger. Osmotically-evoked hypertrophy is dependent on activation of TRPV1 channels, on the firing of APs, on Ca^{2+} influx through L-type Ca^{2+} channels, and on SNARE-dependent exocytotic fusion. We do not know the source of the internal membranes that are responsible for hypertrophy, but it is unlikely to be due primarily to the fusion of neuropeptide-containing granules because osmotically-evoked release of VP from MNC somata is slow (Leng *et al.*, 2008) and because there are not likely to be enough neuropeptide-containing granules to induce such an increase in total membrane area. It therefore appears likely that hypertrophy involves transfer of membrane from a large internal source such as the endoplasmic reticulum, but it could also involve the fusion of specialized membrane vesicles or granules to mediate the translocation of specific membrane proteins to the plasma membrane. We have shown that an osmotically-evoked increase in the activity of PLC is required for the initiation of hypertrophy and that activation of PKC is necessary and sufficient to cause MNC enlargement. It will be interesting to determine the mechanism by which PKC activation triggers membrane transfer to the MNC plasma membrane.

Acute osmotically-evoked changes in MNC size are not associated with changes in membrane capacitance (Zhang *et al.*, 2003b) and thus our observations suggest a novel mechanism for MNC hypertrophy. Although we observed an increase in the mean CSA of MNCs from the shrunken state to the hypertrophied state of about 11% (i. e. from 343 to 381 μm^2), the increase in cell membrane capacitance was only about 7%. The smaller increase in cell capacitance probably reflects the fact that the capacitance measurement includes membrane that

is not on the somatic cell surface, such as that in the MNC processes and in the large membrane reserve that MNCs possess (Zhang *et al.*, 2003b). Increasing the volume of the MNC soma by a given amount would therefore be expected to cause a somewhat lower increase in the total membrane area (and the measured membrane capacitance). Both the measurement of CSA changes and the change in capacitance, however, are markedly lower than the changes evoked by water deprivation or salt loading (see above). The extent of the increase under our conditions may be limited by the time of exposure, by the absence of most of the MNC dendritic tree, or by the absence of signaling molecules that are derived from a cell type that is present *in vivo* but absent from our preparation (e.g. the surrounding astrocytes).

Osmotically-evoked hypertrophy is of particular interest in the MNCs because their osmosensitivity is thought to depend on a stretch-inactivated cation current (Oliet *et al.*, 1993a) mediated by TRPV1 channels (Sharif Naeini *et al.*, 2006) that are activated by the decrease in membrane tension caused by cell shrinkage (Zhang *et al.*, 2003b). The MNCs have been shown to respond to hypertonic saline by shrinking and remaining shrunk for up to 6 minutes, suggesting that they do not display acute cell volume regulation in response to osmotically-evoked cell shrinkage (Zhang *et al.*, 2003b). Our results are consistent with this report because hypertrophy occurs only after a significant delay (see Figure 2.1) and depends on mechanisms distinct from those underlying the acute cell volume regulatory mechanisms observed in many other neuron types. It is important to note, however, that water molecules will always tend to flow in or out of the cell to equalize the internal and external osmolality and therefore the increases in cell volume observed *in vivo* or by us *in vitro* must be accompanied by mechanisms to increase the ionic content of the MNC cytoplasm. The lack of effect of bumetanide suggests that the activity of the Na^+-K^+-Cl^- co-transporter NKCC1 is not required, but the mechanism underlying the increase in

cytoplasmic volume in MNCs remains to be determined.

The increase in MNC membrane during osmotically-evoked hypertrophy has implications on the mechanisms by which TRPV1 channels mediate MNC osmosensitivity. We observed that hypertrophy rapidly reverses when Ca^{2+} influx into the MNCs is suppressed by the block of the TRPV1 channels, Na^{+} channels, or L-type Ca^{2+} channels (see Figure 2.2B). The maintenance of hypertrophy therefore depends on the continuation of AP firing. This suggests either that the addition of new membrane to the MNC plasma membrane does not alter the membrane tension, thereby allowing the TRPV1 channels to continue to be in an active state, or that a different mechanism is involved in maintaining the activity of the TRPV1 channels in MNCs following hypertrophy. It is possible, for example, that TRPV1 channels are regulated both by membrane tension and by one or more signaling molecules (which could include PIP_2) or that hypertrophy leads to an increase in TRPV1 activity by causing translocation of the channels to the MNC plasma membrane.

Although the physiological significance of MNC hypertrophy remains unclear, it is possible that the fusion of internal membranes mediates the translocation of specific channels, receptors, or other membrane proteins to the MNC plasma membrane. This process could be involved, for example, in the dehydration-induced increase in the cell surface expression of V_{1a} vasopressin receptors (Hurbin *et al.*, 2002), Na^{+} currents (Tanaka *et al.*, 1999), dynorphin receptors (Shuster *et al.*, 1999), and L-type Ca^{2+} channels (Zhang *et al.*, 2007a), and the Ca^{2+}-dependent translocation of N-type Ca^{2+} channels (Tobin *et al.*, 2011). The activation of PKC by DAG has been implicated in analogous forms of translocation, including that of Ca^{2+} channels in molluscan neuroendocrine cells (Strong *et al.*, 1987) and of TRPV1 in an oocyte expression system

(Morenilla-Palao *et al.*, 2004), and we therefore tested whether PKC could play a role in triggering MNC hypertrophy. Our data suggest that hypertrophy is dependent upon activation of both PLC and PKC. The activation of PKC is sufficient to activate at least part of the response, although the small size of the response to PKC activator alone may suggest that other triggers, for example intracellular Ca^{2+}, may contribute to the full response. Evidence of whether the hypertrophic response does involve the translocation of channels and receptors awaits further study. PKC-mediated translocation of Ca^{2+} channels or TRPV1 channels could play an important role in MNC osmosensitivity. Ca^{2+} channels have been observed on intracellular granules in MNCs (Fisher *et al.*, 2000) and this could represent an internal pool that is available for translocation to the MNC membrane.

The osmotically-evoked increase in PLC activity could also be important in mediating osmosensitivity by regulating MNC activity in other ways. PIP_2 has been shown to regulate the activity of a large number of ion channels, and in particular both TRP channels and M-type K^+ currents (Suh *et al.*, 2005). The latter is important because we identified an M-type K^+ current in the MNCs (Liu *et al.*, 2005; Zhang *et al.*, 2009). We also showed that this current is suppressed by muscarinic activation (Zhang *et al.*, 2009) and our current data is consistent with the hypothesis that this occurs by the G-protein mediated activation of PLC, as occurs in other neurons (Suh *et al.*, 2005). M-currents are low threshold, slow K^+ currents and their modulation has important effects on the excitability of many central neurons (Brown *et al.*, 2009) and it is possible that they are important in MNC physiology as well. We showed that when MNCs are subjected to whole cell patch clamp and then exposed to an increase in external osmolality, there is an increase in this M-type current (Zhang *et al.*, 2009). My current data show that osmotic activation of PLC decreases PIP_2 and would therefore be expected to decrease the amplitude of the M-type

currents. It is possible that the activity of PLC and/or the regulation of PIP_2 levels is altered during whole cell patch clamp and that our earlier results do not therefore reflect the physiological mechanism of osmotic regulation of M-type current. It is also possible that the M-current is regulated in some way other than by changes in PIP_2. We are currently working to resolve this contradiction.

Our data suggest that osmotically-evoked, activity- and Ca^{2+}-dependent exocytotic fusion may underlie part or all of the hypertrophy observed in MNCs following water deprivation or salt loading. Hypertrophy occurred in response to modest changes in osmolality suggesting that the size of MNCs may be regulated *in vivo* in a dynamic fashion as the electrical activity of the MNCs responds to changes in external osmolality. The full significance of this phenomenon is not clear, but it could represent a mechanism for osmotically-induced translocation of channels and receptors to the MNC plasma membrane and could contribute to the adaptive response of MNCs to sustained high osmolality. Our data suggest that this process is mediated by an activity-dependent increase in PLC activity, leading to an increase in PKC activity. The PLC-mediated decrease in PIP_2 and increase in DAG and IP_3 could also play a number of other important roles in regulating ion channel function in MNCs. Our data therefore have important implications for acute and longer-term osmosensitivity of the MNCs.

Preface to Chapter 3:

The following chapter is a manuscript that was submitted to Journal of Physiology for publication in August 2016. The title and authorship details of this manuscript are as follows:

Osmotic activation of a Ca^{2+}-dependent phospholipase C pathway that regulates TRPV1 currents in rat supraoptic neurons

Bansal V and Fisher TE

Being the first author of this manuscript, I have made few minor modifications like changing the page numbers, figure numbers and rewording of a few sentences in the original manuscript to make it fit in my manuscript based thesis format. My experimental contributions in this manuscript includes doing all the experiments i.e. immunocytochemistry and whole cell patch clamp experiments on rat MNCs that are included in this manuscript. I am therefore responsible for all data that is shown in Figures 1-9 of this manuscript.

In this manuscript, I have characterized the Ca^{2+} dependence of the previously identified osmotically-evoked PLC signalling pathway in rat MNCs using different experimental combinations. Specifically I have shown that osmotically-evoked PLC pathway in rat MNCs is dependent on Ca^{2+} influx that is mediated depolarization-evoked opening of L-type Ca^{2+} channels. I have also demonstrated that this Ca^{2+} dependent PLC pathway can also be activated by high K^+ saline and Ca^{2+} ionophore treatment that could raise intracellular Ca^{2+} levels by non-osmotic methods. I have also shown that this osmotically-evoked PLC pathway acts in a feed forward manner to potentiate the intrinsic osmosensitivity of MNCs, by activating the TRPV1 encoded SICs and is therefore an important event in the osmotic physiology of MNCs.

Also included at the end of this chapter is an appendix (Appendix 3A) that contains data

for some PIP_2 immunocytochemical experiments that I have performed using wild type and PLC δ1 knockout mice. These experiments were performed to test the possibility if the Ca^{2+} dependent PLC isoform that is osmotically activated in the MNCs could be the PLC δ1. The brief rationale, methodology and results obtained from these experiments have been shown in the appendix.

CHAPTER 3

OSMOTIC ACTIVATION OF A CA^{2+}-DEPENDENT PHOSPHOLIPASE C PATHWAY THAT REGULATES TRPV1 CURRENTS IN RAT SUPRAOPTIC NEURONS

3.1 ABSTRACT

The magnocellular neurosecretory cells (MNCs) synthesize vasopressin (VP) and secrete increasing amounts of it as the osmolality of the extracellular fluid increases. MNC osmosensitivity depends on mechanosensitive TRPV1 channels whose level of activation depends on osmotically-induced changes in cell volume. Acute increases in external osmolality decrease PIP_2 levels in the plasma membrane of acutely isolated rat MNCs through the activation of phospholipase C, which catalyzes the breakdown of PIP_2 to IP_3 and diacylglycerol (a protein kinase C activator). I now show that the osmotic activation of PLC is rapid, reversible, and dose-dependent, with the maximal effect requiring an approximately 10% increase in osmolality. This response is absent when external Ca^{2+} is removed and can be mimicked by treatment with a Ca^{2+} ionophore or by high K^+-mediated depolarization in the presence of external Ca^{2+}. PLC activation is prevented by TRPV1 or L-type Ca^{2+} channel antagonists. I used whole cell patch clamp to show that the osmotic activation of TRPV1 current is significantly diminished in the presence of a PLC inhibitor, suggesting that PLC contributes to the osmotic activation of TRPV1 current. The presence of a PIP_2 analogue did not alter osmotic activation of the TRPV1 current, but a PKC inhibitor decreased the response. A PKC activator increased TRPV1 current in isotonic saline and enhanced the osmotic activation of the TRPV1 current. My data therefore suggest that MNCs possess an osmotically-activated Ca^{2+}-dependent PLC that regulates TPRV1 channels and may be important in MNC osmosensitivity and in central osmoregulation.

3.2 INTRODUCTION

The magnocellular neurosecretory cells of the hypothalamus (MNCs) play a critical role in the regulation of the osmolality of body fluids. When the osmolality of the plasma rises, MNCs fire more frequently and release more vasopressin (VP), which decreases urine output and thereby preserves body water, and oxytocin (OT), which, at least in some species, complements the effect of VP by increasing the excretion of Na^+ (Verbalis *et al.*, 1991; Bourque *et al.*, 1997). When the plasma has low osmolality, the release of VP and OT is low, which enables the production of high volumes of dilute urine to rid the body of excess water. The osmosensitivity of the MNCs depends on osmotically-induced changes in cell volume and the resultant changes in the opening probability of stretch-inactivated (SIC) non-selective cation channels (Oliet *et al.*, 1993a; Oliet *et al.*, 1994) that are mediated by a variant of the TRPV1 channel (Sharif Naeini *et al.*, 2006; Zaelzer *et al.*, 2015). Activation of these channels depolarizes the MNCs and makes them more likely to fire APs in response to excitatory inputs from osmosensitive neurons in neighbouring regions (Oliet *et al.*, 1994).

I recently reported that MNCs acutely isolated from the rat supraoptic nucleus (SON) show decreased plasma membrane immunoreactivity to phosphatidylinositol 4, 5-bisphosphate (PIP_2) when exposed to increases in external osmolality and that this effect depends on the activation of phospholipase C (PLC; (Shah *et al.*, 2014). PLC catalyzes the hydrolysis of PIP_2 into two second messengers - diacylglycerol (DAG) and inositol 1, 4, 5-trisphosphate (IP_3; (Rhee, 2001). This reaction could be an important contributor to MNC osmosensitivity because all three of these molecules regulate a variety of cell processes (Suh *et al.*, 2008b) and in particular the function of many types of ion channel (Hilgemann *et al.*, 2001). Ion channels modulated by the activity of PLC include TRPV1 channels (Rohacs *et al.*, 2008) and M-type K^+ channels (Li *et al.*, 2005), which have

also been identified in the MNCs (Zhang *et al.*, 2009).

I therefore sought to characterize the osmotic activation of PLC, determine the mechanism by which it is activated, and determine whether the activation of PLC regulates the activity of TRPV1 currents in MNCs. I now show that the osmotic activation of PLC in MNCs is rapid (occurring within two minutes), reversible, and dose-dependent, with the maximal effect requiring an approximately 10% increase in osmolality. I also show that osmotic activation of PLC is prevented when Ca^{2+} is absent from the external solution and can be mimicked by administration of a Ca^{2+} ionophore or by high K^{+}-mediated depolarization in the presence of external Ca^{2+}. PLC activation is also prevented by antagonists of TRPV1 channels or of L-type Ca^{2+} channels. These data suggest that rat MNCs express a Ca^{2+}-dependent PLC pathway and that this pathway is triggered by an influx of Ca^{2+} through L-type Ca^{2+} channels that is evoked by TRPV1-mediated depolarization. I also used whole cell patch clamp to study the consequences of osmotic activation of PLC on TRPV1 current in the MNCs. I found that the osmotic activation of TRPV1 current is significantly diminished in the presence of a PLC inhibitor, suggesting that PLC enhances the osmotically-evoked activation of these currents. Inclusion of a PIP_2 analogue in the patch pipette did not alter the response to increases in osmolality, suggesting that the PLC-mediated regulation of TRPV1 channels does not depend on the decrease in PIP_2, but rather may depend on a product of PIP_2 hydrolysis such as diacylglycerol (DAG), which acts by activating protein kinase C (PKC). I found that inhibition of PKC suppresses the osmotic activation of TRPV1 current and that treatment with an agent that mimics DAG caused an increase in TRPV1 current is isotonic solution and potentiated the osmotic activation of TPRV1 current. My data therefore suggest that MNCs possess an osmotically-activated PLC that modulates TPRV1 current and may therefore be important in MNC osmosensitivity and in central osmoregulation in mammals.

3.3 MATERIALS AND METHODS

3.3.1 Ethical approval

This work was approved by the University of Saskatchewan's Animal Research Ethics Board, and adhered to the Canadian Council on Animal Care guidelines for humane animal use.

3.3.2 Chemicals

All drugs and chemicals used in the following experiments were purchased from Sigma (St Louis, MO, USA) unless stated otherwise. The PLC inhibitor U73122, the TRPV1 antagonist SB366791, the PKC inhibitor GF-109203 X, and the inactive analogue of the PKC inhibitor were purchased from Enzo Life Sciences, Inc. (Farmingdale, NY, USA). The PIP_2 analogue $PI(4,5)P_2$-diC8 was purchased from Echelon Biosciences Inc. (Salt Lake City, UT, USA).

3.3.3 Animals and cell preparation

Rat MNCs were isolated using a protocol described previously (Liu *et al.*, 2005) and were presumed to be MNCs if they had a maximal cross-sectional area greater than 160 μm^2 (Oliet *et al.*, 1992). In brief, male Long–Evans rats (200–300 g) were anaesthetized with isoflurane and killed by decapitation on the day of experiment. The brain was rapidly removed and blocks of tissue containing most of the two SON were carefully excised. The tissue blocks were then incubated with an oxygenated (100% O_2) Pipes solution (pH 7.1) composed of (in mM): 120 NaCl, 5 KCl, 1 $MgCl_2$, 1 $CaCl_2$, 20 Pipes, 10 glucose and containing trypsin (Type XI, 0.6 mg ml^{-1}) for 90 min at 34°C. After enzymatic treatment with trypsin, the tissue blocks were transferred into the oxygenated Pipes solution (pH 7.4) without trypsin for another 30 min at room temperature. Finally, the tissue blocks were gently triturated with fire-polished pipettes to disperse the MNCs which were then plated onto glass-bottomed culture dishes (Mat Tek) and kept at room temperature for the electrophysiological/immunocytochemical experiments as discussed below.

3.3.4 PIP_2 immunocytochemistry

Acutely isolated rat MNCs plated on glass-bottom dishes were perfused with different experimental solutions for specific time periods as indicated in the text. The osmolalities of all hypertonic solutions used in these experiments were adjusted by adding mannitol and measured using a VAPRO pressure osmometer (WESCOR; Logan, UT, USA). The high K^+ saline was prepared by iso-osmotic substitution of 25 mM NaCl with 25 mM KCL saline of the isotonic Pipes solution. At the end of each experiment, the MNCs were subjected to phosphatidylinositol 4, 5-bisphosphate (PIP_2) immunostaining using a modification of a published protocol (Hammond *et al.*, 2006) that have been used by me previously (Shah *et al.*, 2014). Briefly, the cells were fixed with phosphate-buffered saline (PBS) containing 4% paraformaldehyde for 20–25 min at room temperature. Following three washes with PBS, the cells were blocked with solution containing 10% donkey serum and 0.5% saponin for 1 hr. The cells were then incubated with a mouse monoclonal PIP_2 antibody (Enzo Life Sciences; 1:1000) overnight at 4°C. The next day, dishes were washed with PBS three times and incubated for 1 hour with donkey anti-mouse secondary antibody (Invitrogen Alexa Fluor 488, 1:1000). After three washes with PBS, Citifluor mounting solution (Citifluor Ltd; Gore, QC, Canada) was added to the dishes and cells were then viewed using a Zeiss inverted Axiovert 200 microscope with appropriate filter sets and a 40X objective lens and images were captured using an attached cooled CCD camera. The images were later analyzed using Image-J software (NIH) by tracing the perimeter of each MNC by following the line of greatest fluorescence (disregarding processes) and determining the mean fluorescence of pixels on that line. The mean intensities of staining for all MNCs in each treatment group were then normalized to the mean fluorescence of all the control cells done on each experimental day and the data so obtained was expressed as normalized mean ± SEM for each group. The normalized intensities of

all treatment groups were then statistically compared with the control group values using the student's 'paired t' test. The differences were deemed significant if $P < 0.05$.

3.3.5 Electrophysiological methods

The plated MNCs were maintained in an isotonic (295 mosmol kg^{-1}) external recording solution having (in mM): 140 NaCl, 5 KCl, 10 Hepes, 1 $MgCl_2$, 1 $CaCl_2$, 10 glucose and 0.0005 tetrodotoxin (pH 7.4) and placed on the microscope stage of the electrophysiology station for current measurements using an EPC-9 amplifier (HEKA Elektronik; Lambrecht/Pfalz, Germany) controlled with PULSE software (HEKA). The MNCs were impaled and whole cell patch clamp configuration was achieved by using patch pipettes having resistance of 2-4 MΩ and filled with an internal solution (osmolality 280 mosmol kg^{-1} and pH 7.2) having (in mM): 125 KCl, 10 Hepes, 1 $MgCl_2$, 0.5 EGTA, 4 Na_2-ATP, 1 Na-GTP,14 phosphocreatine unless otherwise stated. The osmolalities of all perfusing solutions were adjusted by adding mannitol. Borosilicate glass capillaries (1.2 mm o.d., 0.68 mm i.d; A-M Systems; Carlsborg, WA, USA) were used to pull patch pipettes on a P-97 horizontal pipette puller (Sutter Instrument Company; Novato, USA) and fire-polished using a microforge (Narashige; Tokyo, Japan). The MNCS were exposed to different experimental treatment conditions as specified in the result section and macroscopic whole cell ramp currents were evoked from a holding potential of -70 mV using a 5 second v-ramp protocol from -100 mV to -20 mV under isotonic conditions. The cells were then switched to current clamp mode and perfused with hypertonic saline solution before re-recording the macroscopic whole cell ramp currents to estimate the effect of different experimental treatments. All currents measurements were made using PULSEFIT software (HEKA) and the signals were low-pass filtered at 2 kHz and digitized at 20 kHz for all experiments. The individual current traces obtained in isotonic and hypertonic conditions for all the MNCs belonging to a particular treatment group were averaged

to generate mean ramp current traces before and after treatment. These traces were then digitally subtracted to obtain the current evoked by each treatment. The mean reversal potentials of the evoked currents were calculated by averaging the individual reversal potentials and were expressed as mean ± SEM. The peak amplitude of the osmotically-evoked ramp current (mean current between -100 and -90 mV at the beginning of the ramp protocol) of all MNCs was also measured and normalized with their respective MNC size by dividing the current value with the whole cell capacitance of the MNC to obtain a peak osmosensitive current density. These individual peak current densities were then pooled and averaged for all treatment groups and expressed as mean ± SEM. I also calculated the osmotically-induced increase in membrane conductance of all MNCs by measuring the difference in the slope values of their I-V plots between -100 and -50 mV (linear region) obtained under isotonic and hypertonic conditions. These increases in conductance were pooled and expressed as mean ± SEM. The mean reversal potentials, averaged peak osmosensitive current density, and mean increase in membrane conductance values for each experimental group were statistically compared with the respective control group values, as detailed in the results, using a student's 'unpaired t' test. The differences were deemed significant if $P < 0.05$.

3.4 Results

3.4.1 Receptor-mediated stimulation of PLC by angiotensin II, direct stimulation of PLC by a PLC activator, and osmotic stimulation of isolated MNCs all decrease membrane PIP_2 to a similar extent

Angiotensin II (Ang II) activates the PLC pathway in rat MNCs through a receptor-dependent mechanism (Chakfe *et al.*, 2000; Zhang *et al.*, 2008). I therefore compared the response to Ang II to the osmotically-evoked activation of PLC in acutely isolated MNCs. I perfused subsets of MNCs

with either hypertonic (325 mosmol kg^{-1}) solution or an isotonic (295 mosmol kg^{-1}) solution containing 5 µM Ang II for 5 minutes each. The mean membrane fluorescence values of hypertonic- and Ang II-treated cells were then compared with the control MNCs that were incubated in isotonic solution. As illustrated in Figure 3.1, exposure to an increase in osmolality of +30 mosmol kg^{-1} caused an approximately 20% decrease in PIP_2 immunoreactivity. Exposure of MNCs to 5 µM Ang II, which would be expected to cause maximal activation of the receptor-linked PLC pathway (Chakfe *et al.*, 2000; Chakfe *et al.*, 2001), caused a decrease of similar magnitude. I co-applied Ang II and hypertonic saline in another group of rat MNCs, but did not see an increase in the response compared to either treatment alone (Figure 3.1). Figure 3.1 also shows that treatment with the non-specific PLC activator m-3M3FBS (30 µM) for 5 minutes caused a decrease in PIP_2 immunoreactivity of similar magnitude, suggesting that the PIP_2 decreases observed may reflect the maximal decrease that is possible through physiological activation of PLC-mediated hydrolysis. The normalized PIP_2 immunoreactivity values observed during the above experiments were as follows: isotonic saline (control; 100.0 ± 8.8; *n* = 191 cells in 5 experiments), hypertonic (325 mosmol kg^{-1}) saline (79.8 ± 6.8; *n* = 148 cells in 5 experiments), isotonic saline containing 5 µM Ang II (82.3 ± 6.9; *n* = 160 cells in 5 experiments), isotonic saline containing 3M3FBS (80.6 ± 9.1; *n* = 157 cells in 5 experiments) and hypertonic saline with 5 µM Ang II (79.0 ± 6.9; *n* = 142 cells in 5 experiments). These results are summarized in the bar graphs in Figure 3.1B. My results suggest that osmotic activation of PLC could contribute to the intrinsic osmosensitivity of the MNCs in ways that overlap with the effects caused by Ang II (Zhang *et al.*, 2008). I therefore tested whether the dose and time dependence of the osmotic activation of PLC are consistent with the intrinsic osmosensitive properties of the MNCs.

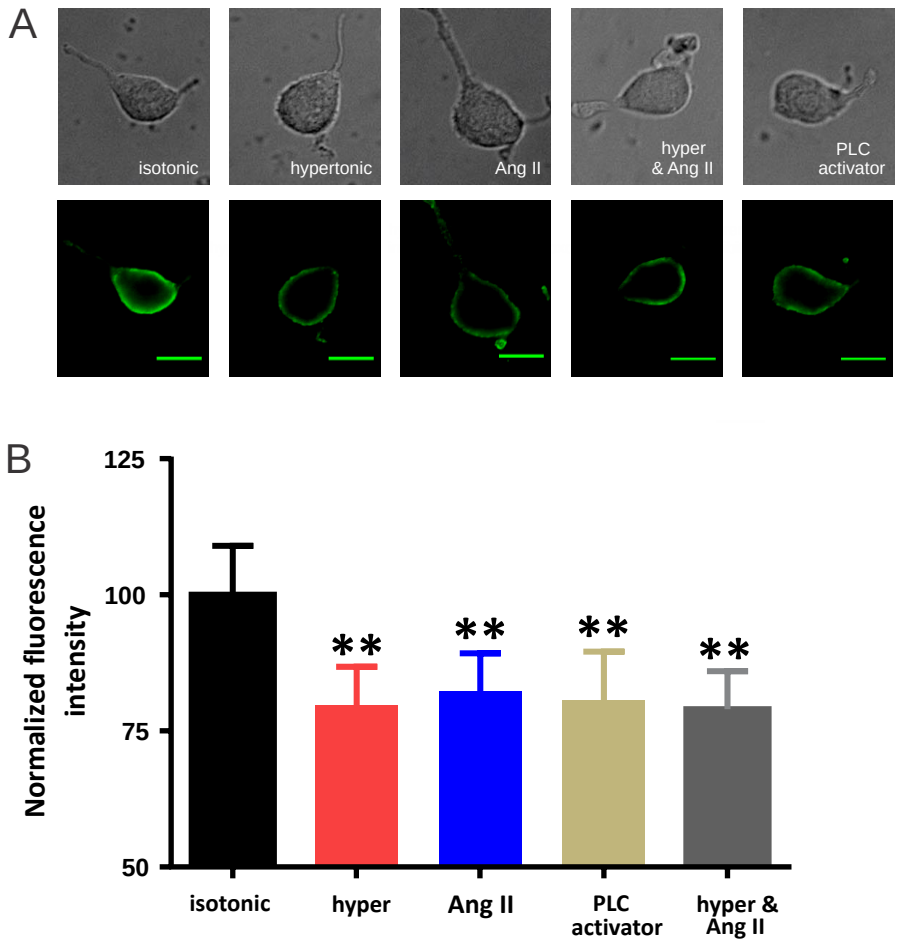

Figure 3.1. Exposure of acutely isolated rat MNCs to hypertonic saline, Ang II, a PLC activator, or hypertonic saline with Ang II all decrease membrane PIP_2 to a similar extent. *A*, images of isolated MNCs using either differential interference contrast images (upper panels) or fluorescence images showing immunoreactivity for PIP_2 (lower panels) maintained in isotonic saline or exposed to hypertonic saline (325 mosmol kg^{-1}), Ang II (5 µM), the PLC activator 3M3FBS (30 µM), or hypertonic saline containing Ang II (5 µM)) for 5 minutes. The scale bar indicates 20 µm. *B*, The bar graph shows the mean normalized immunoreactivity to PIP_2 in the five conditions. Data are expressed as mean normalized fluorescence intensity ± SEM ($P < 0.05$ is indicated by *; $P < 0.01$ by **).

3.4.2 Acute exposure to hypertonic saline causes a reversible, time- and dose-dependent decrease in PIP_2 immunoreactivity

I perfused dishes of isolated MNCs with saline solutions of five different osmolalities (305, 315, 325 and 345 mosmol kg^{-1}) for 5 minutes and then compared their mean membrane fluorescence values with those of the control MNCs that were incubated in isotonic saline (295 mosmol kg^{-1}). As illustrated in Figure 3.2A, the decrease in membrane PIP_2 was found to occur in a dose-dependent fashion and was statistically significant at all osmolalities equal to or higher than 315 mosmol kg^{-1}. The response appeared to be maximal at an osmolality of 325 mosmol kg^{-1} and was not larger at an osmolality of 345 mosmol kg^{-1}. The normalized PIP_2 immunoreactivity observed during these experiments were as follows: 295 mosmol kg^{-1} (control; 100.0 ± 10.7; *n* = 174 cells in 5 experiments), 305 mosmol kg^{-1} (94.4 ± 8.7; *n* = 166 cells in 5 experiments), 315 mosmol kg^{-1} (87.0 ± 9.2; *n* = 155 cells in 5 experiment), 325 mosmol kg^{-1} (78.2 ± 5.9; *n* = 185 cells in 5 experiments) and 345 mosmol kg^{-1} (79.3 ± 6.3; *n* = 161 cells in 5 experiments). Data are expressed as mean normalized fluorescence intensity ± SEM ($P < 0.05$; $P < .01$).

I next sought to determine the time course of the response. I exposed dishes of MNCs to 325 mosmol kg^{-1} saline solutions for four different periods (30 seconds, 2 minutes, 5 minutes and 20 minutes) and compared their mean membrane fluorescence values with those of the control cells that were incubated in isotonic solution. As illustrated in Figure 3.2B, a decrease in membrane PIP_2 was observed within 2 minutes, was maximal at 5 minutes, and persisted for 20 minutes. The mean normalized PIP_2 immunoreactivity values observed were as follows: isotonic (control; 100.0 ± 13.5; *n* = 234 cells in 6 experiments), 30 second exposure (98.4 ± 10.8; *n* = 155 cells in 6 experiments), 2 minute exposure (84.9 ± 10.7; *n* = 177 cells in 6 experiments), 5 minute exposure (74.2 ± 10.9; *n* = 222 cells in 6 experiments) and 20 minute exposure (80.6 ± 9.2; *n* = 188

cells in 6 experiments) to hypertonic saline (325 mosmol kg^{-1}).

I next tested if the osmotically-evoked decrease in PIP_2 could be reversed by re-exposure to isotonic saline solution. I perfused dishes of MNCs with 325 mosmol kg^{-1} saline for 5 minutes and then replaced the solution with 295 mosmol kg^{-1} solution for another 5 minutes. As illustrated in Figure 3.2C, PIP_2 immunoreactivity returned to near the control levels 5 minutes after the return to isotonic saline. The normalized PIP_2 immunoreactivity values were as follows: isotonic (control; 100.0 ± 11.2; *n* = 228 cells in 6 experiments), hypertonic for 5 minutes (72.5 ± 9.1; *n* = 161 cells in 6 experiments) and hypertonic for 5 minutes followed by a 5 minute isotonic re-exposure (90.2 ± 10.6; *n* = 189 cells in 6 experiments). The rapid activation and reversibility of the osmotically-evoked PLC effect is consistent with a role for this effect in the intrinsic osmosensitivity of the MNCs.

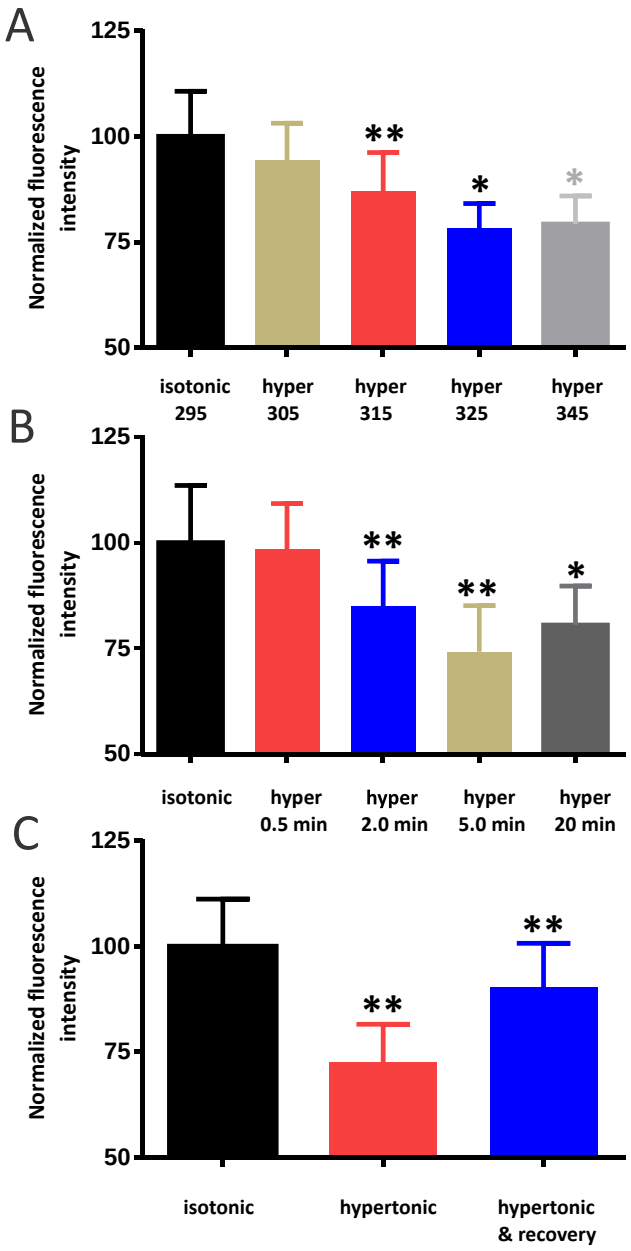

Figure 3.2. Exposure to hypertonic saline causes a dose-dependent, time-dependent, reversible decrease in immunoreactivity to PIP_2 in the plasma membrane of isolated MNCs

A, The bar graph shows the mean normalized immunoreactivity to PIP_2 in MNCs maintained in isotonic saline or hypertonic saline of 305, 326, 325, and 345 mosmol kg^{-1} for 5 minutes. B, The bar graph shows the mean normalized immunoreactivity to PIP_2 in MNCs maintained in isotonic saline or exposed to hypertonic saline (325 mosmol kg^{-1}) for the indicated times. C, The bar graph shows the mean normalized immunoreactivity to PIP_2 in MNCs maintained in isotonic saline, after a 5 minute exposure to hypertonic saline (325 mosmol kg^{-1}), and after 5 minutes of isotonic re-exposure following an initial 5 minutes hypertonic saline exposure. The values for the "hypertonic & recovery" MNCs were compared to those of the "hypertonic" MNCs. Data are expressed as mean normalized fluorescence intensity ± SEM ($P < 0.05$ is indicated by *; $P < 0.01$ by **).

3.4.3 The osmotically-evoked decrease in membrane PIP_2 is dependent on the osmotic activation of TRPV1 channels and on the influx of extracellular Ca^{2+}

The osmotically-evoked excitation of MNCs depends on the activation of TRPV1 channels (Sharif Naeini *et al.*, 2006; Zaelzer *et al.*, 2015), which are mechanosensitive (Oliet *et al.*, 1993a). I therefore exposed dishes of MNCs to either 325 mosmol kg^{-1} saline containing vehicle (DMSO) or 325 mosmol kg^{-1} saline containing 5 μM of the specific TRPV1 channel antagonist SB366791 (which blocks TRPV1 channels in MNCs; (Sharif-Naeini *et al.*, 2008; Sudbury *et al.*, 2013). As illustrated in Figure 3.3A, the osmotically-evoked decrease in membrane PIP_2 was effectively prevented by SB366791, which suggests that the osmotically-evoked PIP_2 decrease is dependent on the activation of TRPV1 channels. The mean normalized PIP_2 immunoreactivity values observed during these experiments were as follows: isotonic saline (control; 100.0 ± 10.7; *n* = 169 cells in 5 experiments), hypertonic saline containing DMSO (81.7 ± 9.7; *n* = 151 cells in 5 experiments) and hypertonic saline containing SB366791 (97.3 ± 10.6; *n* = 172 cells in 5 experiments).

Ca^{2+} influx has been shown to activate PLC pathways in other cell types (Lukacs *et al.*, 2013; Borbiro *et al.*, 2015) and I therefore hypothesized that Ca^{2+} influx could be the trigger for the osmotically-evoked activation of PLC. I therefore treated subsets of MNCs for 5 minutes with either hypertonic saline (325 mosmol kg^{-1}) or hypertonic saline made with no added Ca^{2+}. The mean membrane fluorescence values of all cells were then compared with the control cells that were incubated in isotonic saline. As illustrated in Figure 3.3B, the osmotically-evoked decrease in membrane PIP_2 was effectively eliminated in the absence of external-Ca^{2+}. The mean normalized PIP_2 immunoreactivity values in these experiments were as follows: isotonic saline (control; 100.0 ± 21.2; *n* = 205 cells in 5 experiments), hypertonic saline (70.2 ± 18; *n* = 198 cells in 5 experiments)

and hypertonic saline with no added Ca^{2+}- (105.3 ± 28.54; *n* = 147 cells in 5 experiments). These results suggest that osmotic activation of PLC is a Ca^{2+} dependent process.

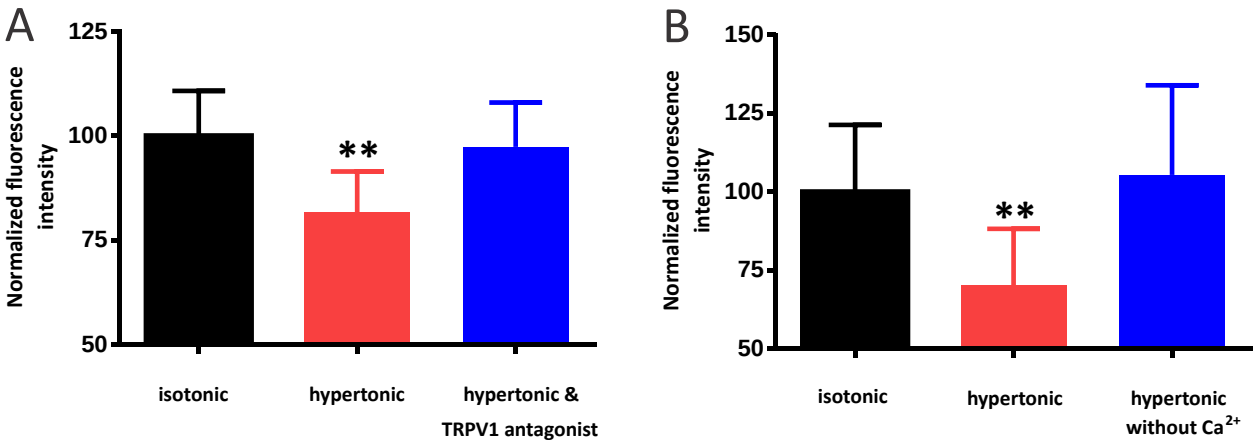

Figure 3.3. The osmotically-evoked decrease in membrane PIP_2 depends on the activation of TRPV1 channels and on extracellular Ca^{2+}

A, The bar graph shows the mean normalized immunoreactivity to PIP_2 in MNCs maintained in isotonic saline, exposed to hypertonic (325 mosmol kg^{-1}) saline for 5 minutes, and exposed to hypertonic saline in the presence of the TRPV1 channel antagonist SB366791 (5 µM) for 5 minutes. B, The bar graph shows the mean normalized immunoreactivity to PIP_2 in MNCs maintained in isotonic saline, exposed to hypertonic (325 mosmol kg^{-1}) saline for 5 minutes, and exposed to hypertonic saline that contains no added Ca^{2+} for 5 minutes. Data are expressed as mean normalized fluorescence intensity ± SEM ($P < 0.05$ is indicated by *; $P < 0.01$ by **).

3.4.4 The osmotically-evoked activation of PLC depends on Ca^{2+} influx through L-type Ca^{2+} channels

Osmotic-dependent Ca^{2+} influx could occur through the TRPV1 channels themselves, which are Ca^{2+} permeable (Zhang *et al.*, 2006), or it could depend on TRPV1-mediated depolarization and the activation of voltage-dependent Ca^{2+} channels, several of which are expressed in MNCs (Fisher *et al.*, 1995; Foehring *et al.*, 1996). I used the Na^+ channel antagonist tetrodotoxin (TTX) to test whether the response requires the firing of APs. Figure 3.4A shows that the inclusion of 0.5 μM TTX during a 5 minute hypertonic treatment did not significantly alter the osmotically-induced reduction in PIP_2, suggesting that under our conditions the firing of APs is not necessary to activate this response. The normalized PIP_2 immunoreactivity values observed were as follows: isotonic saline (control; 100.0 ± 13.6 *n* = 172 cells in 5 experiments), hypertonic (325 mosmol kg^{-1}) saline (73.4 ± 11.1; *n* = 176 cells in 5 experiments), and hypertonic saline in the presence of 0.5 μM TTX (76.9 ± 10.5; *n* = 147 cells in 5 experiments). The inclusion of 30 μM of the L-type Ca^{2+} channel antagonist nifedipine, however, effectively blocked the response (Figure 3.4B), suggesting that Ca^{2+} influx through L-type Ca^{2+} channels is required. The normalized PIP_2 immunoreactivity values observed during these experiments were as follows: isotonic saline (control; 100.0 ± 13.2 n = 228 cells in 6 experiments), hypertonic saline containing DMSO (74.9 ± 12.5; n = 243 cells in 6 experiments), and hypertonic saline containing 30 μM nifedipine (101.3 ± 13.6; n = 160 cells in 6 experiments). Figure 3.5A shows that exposure of the MNCs to an isotonic high K^+ (30 mM) saline (prepared by iso-osmotic substitution of 25 mM NaCl with 25 mM KCl) causes a decrease in membrane PIP_2 and that the decrease is prevented by the L-type Ca^{2+} channel antagonist felodipine (30 μM) or the PLC inhibitor U-73122 (1 μM) or by removal of Ca^{2+} from the external solution. Note that in the high K^+ experiments, 0.5 μM TTX was also added to ensure that the cells

were not firing APs. The mean normalized PIP_2 immunoreactivity values observed during these experiments were as follows: isotonic saline (control; 100.0 ± 11.6; n = 156 cells in 5 experiments), high K^+ isotonic saline (77.4 ± 9.5; n = 168 cells in 5 experiments), high K^+ isotonic saline in the presence of 30 µM felodipine (94.4 ± 9.7 ; n = 150 cells in 5 experiments), high K^+ isotonic saline following a pre-treatment with U-73122 (95.5 ± 12.8; n =141 cells in 5 experiment), and high K^+ saline with no added Ca^{2+} (97.8 ± 13.2; n = 108 cells in 5 experiments). These experiments show that PLC can be activated by high K^+-induced depolarization and the consequent influx of Ca^{2+} through L-type Ca^{2+} channels. These data also suggest that the influx of Ca^{2+} through TRPV1 channels is not necessary for the activation of PLC, but the influx through L-type Ca^{2+} channels is necessary whether cell depolarization is mediated by the activation of TRPV1 receptors or by an increase in the concentration of external K^+. Figure 3.5B shows that treatment with the Ca^{2+} ionophore A23187 (20 µM) causes a decrease in PIP_2 immunoreactivity that is similar to that observed with either hypertonic treatment or exposure to high K^+ saline. The normalized PIP_2 immunoreactivity values observed during these experiments were as follows: isotonic saline (control; 100.0 ± 9.5; n = 183 cells in 5 experiments), hypertonic (325 mosmol kg^{-1}) saline (80.5 ± 7.5; n = 152 cells in 5 experiments), high K^+ isotonic saline (82.4 ± 10.0; n =170 cells in 5 experiments), and isotonic saline containing 20 µM A23187 (79.9 ± 9.1; n = 137 cells in 5 experiments). These data support the hypothesis that Ca^{2+} influx is necessary and sufficient to activate PLC.

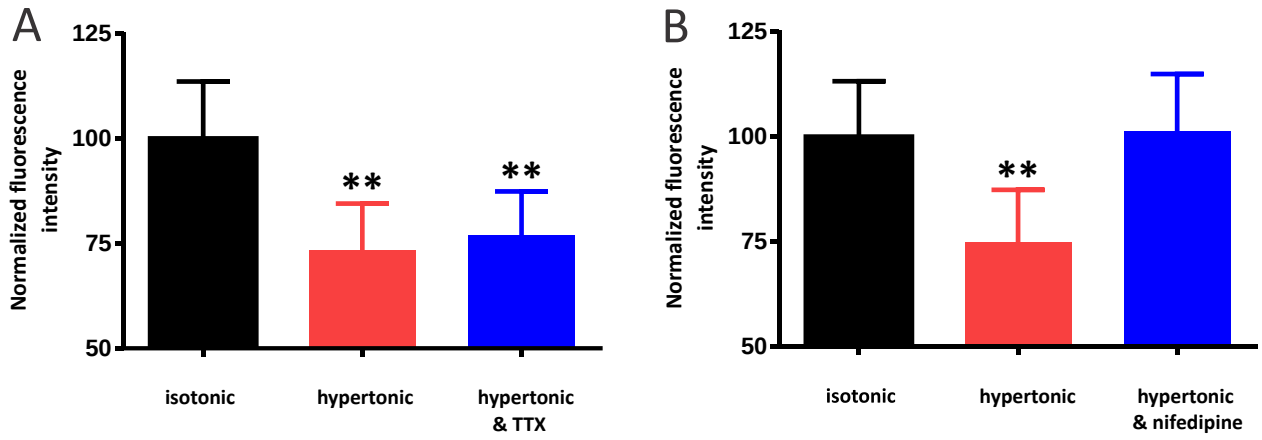

Figure 3.4. The osmotically-evoked decrease in membrane PIP_2 does not depend on action potential firing, but does depend on Ca^{2+} influx through L-type Ca^{2+} channels

A, The bar graph shows the mean normalized immunoreactivity to PIP_2 in MNCs maintained in isotonic saline, exposed to hypertonic saline (325 mosmol kg^{-1}) for 5 minutes, and exposed to hypertonic saline (325 mosmol kg^{-1}) for 5 minutes in the presence of the Na^+ channel antagonist tetrodotoxin (TTX; 0.5 µM). B, The bar graph shows the mean normalized immunoreactivity to PIP_2 in MNCs maintained in isotonic saline, exposed to hypertonic saline (325 mosmol kg^{-1}) for 5 minutes, and exposed to hypertonic saline (325 mosmol kg^{-1}) for 5 minutes in the presence of the L-type Ca^{2+} channel antagonist nifedipine (30 µM). Data are expressed as mean normalized fluorescence intensity ± SEM ($P < 0.05$ is indicated by *; $P < 0.01$ by **).

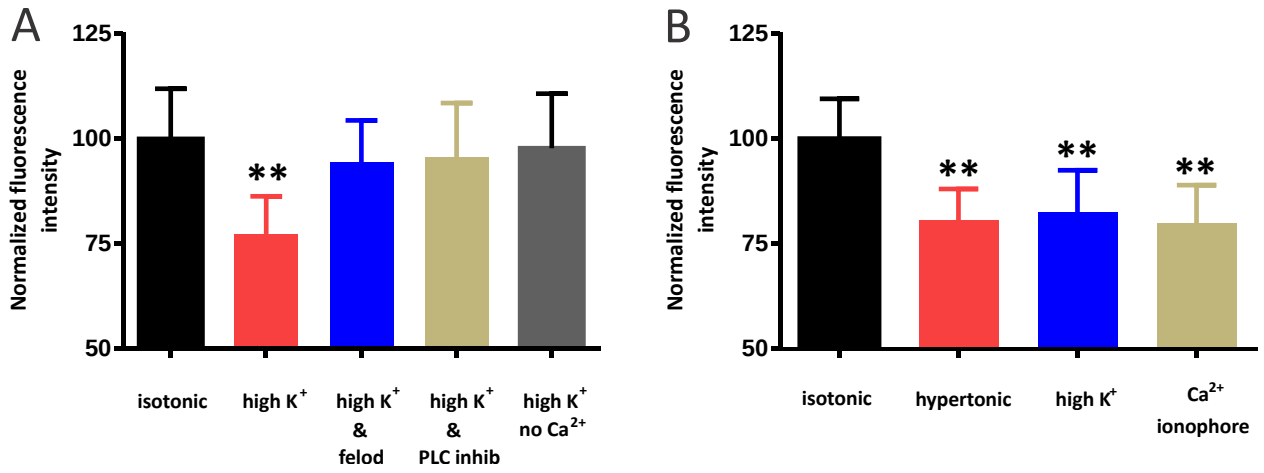

Figure 3.5. The osmotically-evoked decrease in membrane PIP_2 can be mimicked by high K^+-induced Ca^{2+} influx though L –type Ca^{2+} channels or by a Ca^{2+} ionophore

A, The bar graph shows the mean normalized immunoreactivity to PIP_2 in MNCs maintained in isotonic saline, exposed to high K^+ (30 mM) saline for 5 minutes, exposed to high K^+ saline in the presence of the L-type Ca^{2+} channel antagonist felodipine (30 μM) for 5 minutes, exposed to high K^+ saline in the presence of the PLC inhibitor U-73122 (1 μM) for 5 minutes, and exposed to high K^+ saline that contains no added Ca^{2+} for 5 minutes. *B*, The bar graph shows the mean normalized immunoreactivity to PIP_2 in MNCs maintained in isotonic saline, exposed to hypertonic saline (325 mosmol kg^{-1}) for 5 minutes, exposed to high K^+ saline for 5 minutes, and exposed to the Ca^{2+} ionophore A23187 (20 μM) for 5 minutes. Data are expressed as mean normalized fluorescence intensity ± SEM ($P < 0.05$ is indicated by *; $P < 0.01$ by **).

3.4.5 Osmotic activation of PLC contributes to the osmotic activation of TRPV1 currents

MNC excitability is enhanced by treatment with muscarine (Ghamari-Langroudi *et al.*, 2004) or Ang II (Yang *et al.*, 1992; Chakfe *et al.*, 2000), both of which act at G-protein coupled receptors that lead to the activation of PLC (Oude Weernink *et al.*, 2007). Although the mechanism by which muscarine increases excitability is not clear (Ghamari-Langroudi *et al.*, 2004), Ang II has been shown to mediate its stimulatory effect by increasing TRPV1 currents in a PLC-dependent fashion (Chakfe *et al.*, 2000; Zhang *et al.*, 2008). I therefore hypothesized that osmotic activation of PLC could also contribute to the activation of TRPV1 currents. I tested this possibility by using whole cell patch clamp techniques to measure ramp currents (from -100 mV to -20 mV) in MNCs that were bathed in isotonic saline with DMSO or MNCs that were pre-treated with the PLC inhibitor U-73122 (1 μM) for 20 minutes. After stable ramp currents were obtained, the isotonic saline was replaced with hypertonic (345 mosmol kg^{-1}) saline and the ramp currents were recorded again. The traces in the upper part of Figure 3.6A are the means of the ramp currents for cells before and after the addition of hypertonic solution. The traces in the lower panels are the result of digital subtraction of the two currents and therefore show the osmotically-evoked currents in the two groups of cells. The increase in osmolality activated a current with a mean reversal potential of about -30 mV (Figure 3.6A), which is consistent with what has been shown for TRPV1 currents in the MNCs (Voisin et al., 1999; Zhang et al., 2007). The reversal potentials in the vehicle control group (-29.9 ± 2.3 mV) and the PLC inhibitor group (-34.0 ± 3.1 mV) were not statistically different. Figure 3.6 shows that the osmotically-evoked currents were significantly smaller in the presence of the PLC inhibitor, however, both in terms of the increase in conductance and in the peak current density of the osmotically-evoked current. Figure 3.6B shows that the mean increase in membrane conductance in the PLC inhibitor group (1.98 ± 0.25

nS; *n* = 7 cells) was significantly lower than in the control group (4.34 ± 0.68 nS; *n* = 7 cells) and Figure 3.6C shows that the average peak ramp current density in the PLC inhibitor group (8.5 ± 1.2 pA/pF; *n* = 7 cells) was significantly lower than in the control group (16.3 ± 2.1 pA/pF; *n* = 7 cells). These data suggest that PLC contributes to the activation of TRPV1 currents that is observed in response to elevations in external osmolality.

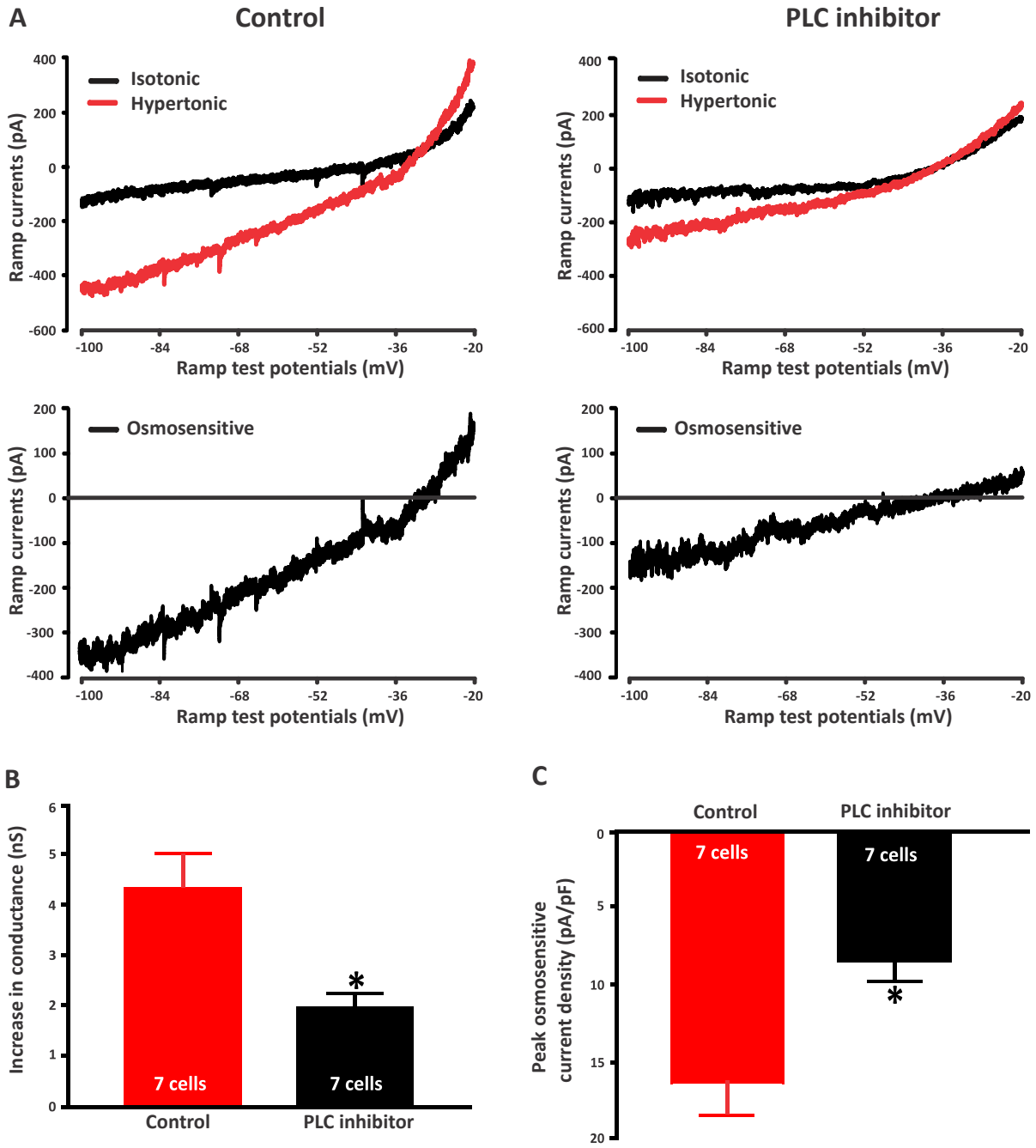

Figure 3.6. PLC Inhibition reduces osmotically-evoked TRPV1 currents

A. The upper panels show the mean ramp current traces evoked before (black trace) and after (red trace) exposure to hypertonic (345 mosmol kg^{-1}) in the absence (left traces) and presence (right traces) of the PLC inhibitor U73122 (1 μM). The lower traces show the digital subtraction of the black and red traces, which therefore represents the osmotically-evoked current under the two conditions. B. The bar graphs show the mean osmotically-evoked increase in the membrane conductance of MNCs in the two conditions. C. The bar graphs show the mean peak densities of osmotically-evoked currents in the two conditions. Data are expressed as mean ± SEM ($P < 0.05$ is indicated by *; $P < 0.01$ by **).

3.4.6 The PLC-mediated enhancement of TRPV1 current depends on the activation of PKC

The observation that inhibition of PLC results in a decrease in the osmotic activation of TRPV1 current suggests that TRPV1 channels are either inhibited by PIP_2 (and thus are enhanced by a decrease in PIP_2) or are activated by one or both downstream products of PLC (IP_3 and DAG). I therefore tested whether inclusion of the water-soluble PIP_2 analogue PIP_2-diC8 (100 μM) in the recording patch pipette would inhibit the osmotic activation of the TRPV1 current. PIP_2-diC8 has been shown to mimic the effects of PIP_2 on ion channels in other cell types (Lukacs *et al.*, 2007; Albert *et al.*, 2008) and its presence should compensate for any evoked decreases in PIP_2 concentration. The average ramp current traces obtained under isotonic and hypertonic (325 mosmol kg^{-1}) conditions for both control and PIP_2-diC8-loaded MNCs are shown in the top panels of Figure 3.7A and the digitally-subtracted average osmotically-evoked currents are shown in the bottom panels. The mean reversal potential of the osmotically-evoked currents in control (-34.1 ± 2.8 mV) and PIP_2-diC8-loaded MNCs (-30.1 ± 4.3 mV) were not statistically different. As illustrated in Figure 3.7B, the mean peak ramp current densities observed in PIP_2 loaded MNCs (11.5 ± 0.4 pA/pF; *n* = 7 cells) was not significantly different from those of the control MNCs (11.9 ± 0.7 pA/pF; *n* = 8). The mean increase in membrane conductance of the PIP_2-diC8-loaded MNCs (3.28 ± 0.38 nS; *n* = 7 cells) was also not significantly different from the control MNCs (3.04 ± 0.21 nS; *n* = 8 cells), as is shown in Figure 3.7C. These data suggest that the effect of PLC activation on TRPV1 currents does not depend on a decrease in the concentration of PIP_2.

I then pre-treated MNCs with the PKC inhibitor GF109203X (2 μM) to test whether PKC activation is involved in the osmotic activation of TRPV1 currents. The ramp currents were measured before and after exposure to hypertonic solution as described above and were

compared to the currents in cells pre-treated with 2 μM of the inactive PKC inhibitor analogue bisindolylmaleimide V. The mean ramp current traces obtained under isotonic and hypertonic (325 mosmol kg^{-1}) conditions are shown in the top panels of Figure 3.8A and the digitally-subtracted mean osmotically-evoked currents are shown in the bottom panels. The mean reversal potential of the osmotically-evoked currents obtained in the inactive analogue (-33.6 ± 2.4 mV) and the PKC inhibitor (-31.3 ± 2.7 mV) were not statistically different. The mean peak ramp current densities in the PKC inhibitor (6.3 ± 0.3 pA/pF; *n* = 9 cells) were significantly smaller than those in the inactive analogue (11.4 ± 1.0 pA/pF; *n* = 5 cells) as well as that found for the control group (11.9 ± 0.7 pA/pF; *n* = 8) in the previous experiment. The mean increase in membrane conductance in the PKC inhibitor (1.99 ± 0.09 nS; *n* = 9 cells) was also significantly smaller than in the inactive analogue (3.16 ± 0.34 nS; *n* = 5 cells) as well as that observed in the control group (3.04 ± 0.21 nS; *n* = 8 cells) from the last experiment (Figure 3.8C). The mean peak ramp current density and the mean increase in cellular conductance observed in the MNCs treated with the inactive PKC analogue were not different than those obtained from the control group in the previous experiment. These data suggest that the activation of PLC contributes to the osmotically-evoked increase in TRPV1 currents through a PKC-dependent mechanism.

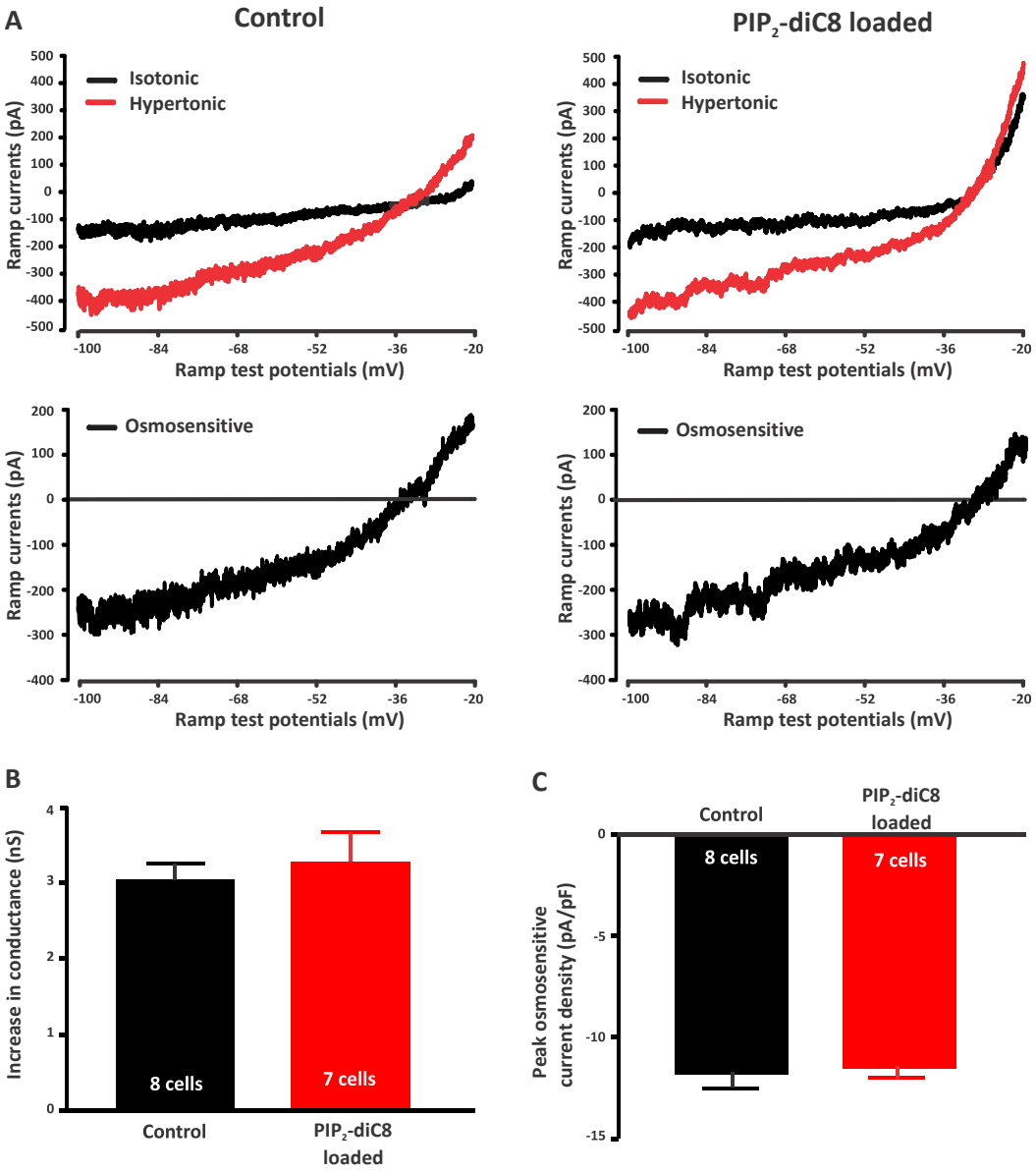

Figure 3.7. A PIP_2 analogue does not affect the osmotic activation of TRPV1 currents
A. The upper panels show the mean ramp current traces evoked before (black trace) and after (red trace) exposure to hypertonic (325 mosmol kg^{-1}) in the absence (left traces) and presence (right traces) of the PIP_2 analogue PIP_2-diC8 (100 μM) in the patch pipette. The lower traces show the digital subtraction of the black and red traces, which therefore represents the osmotically-evoked current under the two conditions. B. The bar graphs show the mean osmotically-evoked increase in the membrane conductance of MNCs in the two conditions. C. The bar graphs show the mean peak densities of osmotically-evoked currents in the two conditions. Data are expressed as mean ± SEM ($P < 0.05$ is indicated by *; $P < 0.01$ by **).

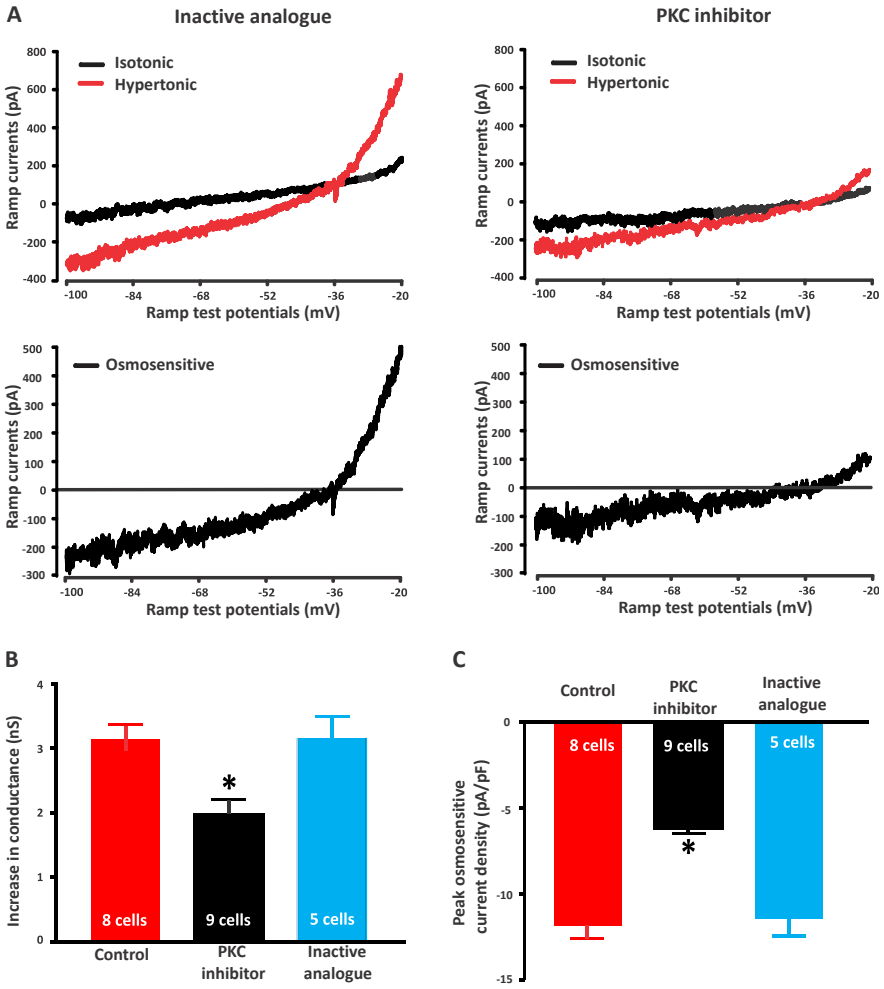

Figure 3.8. PKC inhibition suppresses the osmotic activation of TRPV1 currents
A. The upper panels show the mean ramp current traces evoked before (black trace) and after (red trace) exposure to hypertonic (325 mosmol kg^{-1}) in the presence of an inactive analogue (left traces) and the PKC inhibitor GF109203X (2 μM). The lower traces show the digital subtraction of the black and red traces, which therefore represents the osmotically-evoked current under the two conditions. B. The bar graphs show the mean osmotically-evoked increase in the membrane conductance of MNCs in the three conditions. C. The bar graphs show the mean peak densities of osmotically-evoked currents in the three conditions. Data are expressed as mean ± SEM ($P < 0.05$ is indicated by *; $P < 0.01$ by **).

3.4.7 A PKC activator increases basal TRPV1 currents and also enhances the osmotic activation of TRPV1 currents

The observation that inhibition of PKC suppresses the osmotic activation of TRPV1 currents suggests that PKC may regulate TRPV1 channel activity. I therefore tested whether PKC activation would alter TRPV1 currents in MNCs in isotonic solution and whether PKC activation would alter the osmotic activation of TRPV1 currents. The mean ramp current traces obtained before and after adding the PKC activator phorbol 12-myristate 13-acetate (PMA; 0.2 μM; upper traces) and the digital subtraction of the pre- and post-treatment traces (lower trace) are shown in Figure 3.9A. The PKC activator caused a significant increase in the mean membrane conductance (0.59 ± 0.08 nS; *n* = 7 cells) and in the peak ramp current density (2.7 ± 0.3 pA/pF; *n* = 7 cells), as is shown in Figure 3.9C (left) and Figure 3.9D (left). Another group of MNCs were treated with 4α-PMA (0.2 μM), an inactive analogue of the PKC activator, which caused no significant increase in mean membrane conductance (0.06 ± 0.07 nS; *n* = 5 cells) or in the peak ramp current density (0.8 ± 0.2 pA/pF; *n* = 5 cells), as is shown in the left parts of Figure 3.9C and D (traces not shown). The mean reversal potential of the PKC-sensitive currents (-34.3 ± 2.3 mV) was not statistically different from the osmotically-evoked currents suggesting that the PKC activator is increasing the TRPV1 current.

I tested whether PKC activation would alter the osmotic activation of TRPV1 currents by treating MNCs with PMA (0.2 μM) and then exposing them to hypertonic saline (325 mosmol kg^{-1}). The mean ramp current traces obtained before and after the switch to the hypertonic saline (upper traces) and the digital subtraction of the pre- and post-treatment traces (lower trace) are shown in Figure 3.9B. Hypertonic saline in the presence of the PKC activator caused a significantly

greater increase in mean membrane conductance (8.60 ± 0.39 nS; *n* = 5 cells) than it did in the cells in the previous experiments, which were treated with hypertonic saline in the absence of the activator (3.04 ± 0.21 nS; *n* = 8 cells) and a significantly greater increase in the mean peak ramp current density (29.4 ± 3.9 pA/pF; *n* = 5 cells) than in the previous experiment (11.9 ± 0.7 pA/pF; *n* = 8 cells), as is shown in Figure 3.9C (right) and Figure 3.9D (right). The mean reversal potential of the osmosensitive current in the presence of the PKC activator was -34.6 ± 2.8 mV and was not statistically different from my previously recorded osmotically-evoked currents. These data suggest that PKC activation enhances the osmotic activation of TRPV1 currents in the MNCs.

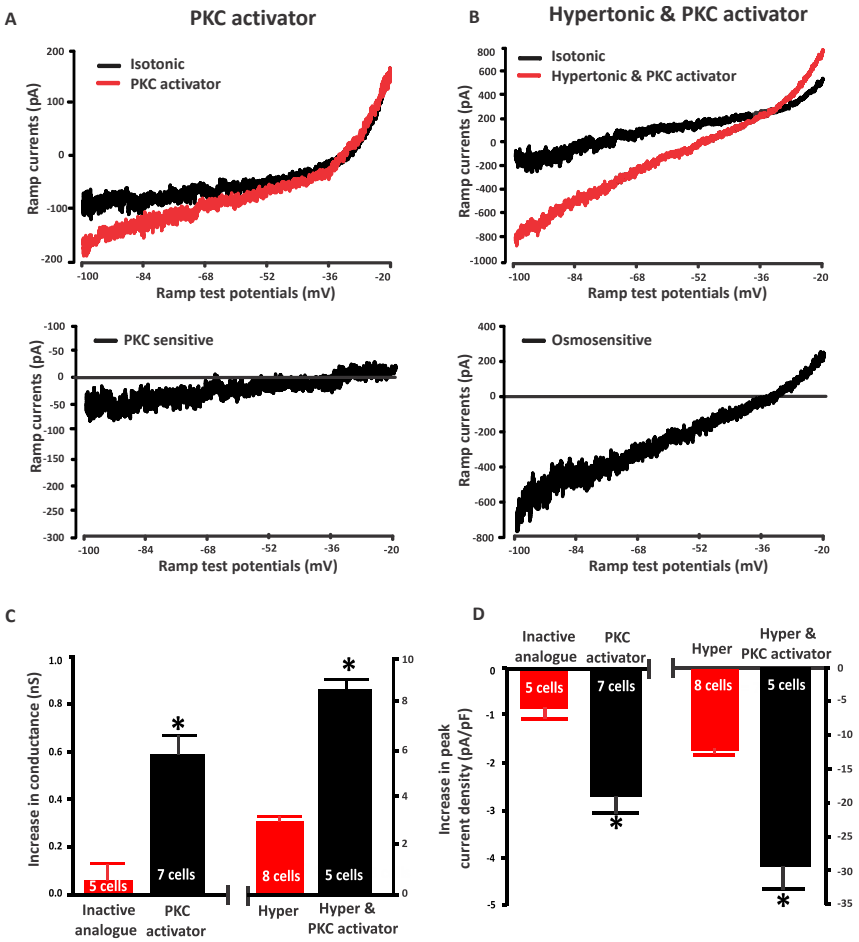

Figure 3.9. PKC activation increases TRPV1 currents in isotonic saline and enhances the osmotic activation of TRPV1 currents

A. The upper panel shows the mean ramp current traces evoked before (black trace) and after (red trace) exposure to the PKC activator phorbol 12-myristate 13-acetate (PMA; 0.2 µM) in isotonic saline. The lower trace shows the digital subtraction of the black and red traces, which therefore represents the PKC activated current. B. The upper panels on the right show the mean ramp current traces evoked before (black trace) and after (red trace) exposure to hypertonic saline (325 mosmol kg^{-1}) in the presence of the PKC activator phorbol 12-myristate 13-acetate (PMA; 0.2 µM). The lower trace shows the digital subtraction of the black and red traces, which therefore represents the osmotically-evoked current in the presence of PMA. C. The bar graph on the left shows the mean increase in the membrane conductance of MNCs caused by the PKC activator and the bar graph on the right shows the effect of hypertonic saline (325 mosmol kg^{-1}) in the presence of the PKC activator compared to the effect of hypertonic saline (325 mosmol kg^{-1}) in the absence of the PKC activator (data from the previous experiment). D. The bar graph on the left shows the mean membrane conductance of MNCs in the presence of the inactive analogue and the PKC activator and the bar graph on the right shows the effect of hypertonic saline (325 mosmol kg^{-1}) in the presence of the PKC activator compared to the effect of hypertonic saline (325 mosmol kg^{-1}) in the absence of the PKC activator (data from the previous experiment). Data are expressed as mean ± SEM ($P <$ 0.05 is indicated by *; $P < 0.01$ by **).

3.4.8 DISCUSSION

My results suggest that the MNCs express an osmotically-evoked Ca^{2+}-dependent PLC pathway that may be important in mediating the electrophysiological changes that MNCs undergo as a consequence of increases in osmolality. I observed an approximately 20% decrease in the immunoreactivity to PIP_2 in the plasma membrane of acutely isolated MNCs in response to a brief exposure to hypertonic saline (325 mosmol kg^{-1}). Although we have no direct evidence that a 20% decrease in membrane PIP_2 and the presumed increases in IP_3 and DAG are functionally relevant, the observation that a similar decrease is evoked by treatment with a physiologically relevant concentration of Ang II (5 μM) supports the hypothesis that a 20% decrease in membrane PIP_2 immunoreactivity indicates a functionally important activation of PLC. Furthermore, the observation that the decrease was not greater when the cells were treated with Ang II and hypertonic saline simultaneously or when the cells were treated with a non-selective PLC activator suggests that the 20% decrease may reflect the response to a strong physiological activation of PIP_2, perhaps even a maximal response under our conditions.

I observed a significant decrease in membrane PIP_2 with a change from 295 mosmol kg^{-1} to 315 mosmol kg^{-1} saline and a larger effect with a change to 325 mosmol kg^{-1}. The decrease was not however larger when the cells were treated with 345 mosmol kg^{-1} saline suggesting that the effect is maximized with an increase to 325 mosmol kg^{-1} saline. This represents an increase in osmolality of about 10%, which is consistent with a role for the osmotic activation of PLC in physiological regulation of MNC function. The minimum increase in osmolality required to evoke a PIP_2 decrease response (+20 mosmol kg^{-1}) is however much greater than that required to observe a change in TRPV1 channel opening (+3 mosmol kg^{-1}; (Oliet *et al.*, 1994). The sensitivity

of my immunocytochemical protocol may underestimate the sensitivity of the PLC system. My protocol requires comparisons between large populations of treated and untreated MNCs and the variability of measurements of PIP_2 immunoreactivity (due in part to differences in cell size and shape) may make it difficult to detect small changes in PIP_2 concentration. Live cell measurements of PIP_2 might give a better estimate of the sensitivity of the response, but this would require transfection of the MNCs with PIP_2-sensitive probes (Lukacs *et al.*, 2013; Borbiro *et al.*, 2015), which is difficult to achieve in acutely isolated adult central neurons (Karra *et al.*, 2010). I have also shown that the osmotic activation of PLC occurs following an exposure to hypertonic saline of two minutes or less and reaches its maximum in about five minutes. For the same reason as mentioned above, my method makes it difficult to see the small changes in PIP_2 levels that would truly reflect the minimum time of exposure to elicit a response. It is possible that changes in PIP_2 concentration occur in less than 2 minutes. The decrease in PIP_2 was reversible and the levels had returned to near the control levels five minutes after a return to an isotonic solution. These observations are consistent with the hypothesis that the osmotic activation of PLC could contribute to MNC osmosensitivity.

I next sought to determine the mechanism by which increases in osmolality stimulate PLC and found that the effect depends on activation of TRPV1 channels and the presence of external Ca^{2+}. In Figure 3.3 I presented data showing that the administration of hypertonic solution in the presence of the TRPV1 channel antagonist SB366791 or in the absence of extracellular Ca^{2+} was unable to decrease the PIP_2 concentration, suggesting that Ca^{2+} influx is necessary for the effect. In Figure 3.4, I presented data showing that the osmotic activation of PLC is not prevented by the Na^+ channel antagonist TTX, but is prevented by the presence of L-type Ca^{2+} channel blocker nifedipine. This suggests that under our conditions acutely isolated rat MNCs do not require APs

to evoke sufficient Ca^{2+} influx to activate PLC, but do require the influx of Ca^{2+} through L-type Ca^{2+} channels.

I also treated MNCs with high K^+ (30 mM) saline, which would be expected to depolarize the MNCs and thereby activate voltage dependent Ca^{2+} channels, and treatment with the Ca^{2+} ionophore A23187 (20 μM), which would directly activate Ca^{2+} influx. Both of these treatments caused a decrease in PIP_2 levels that was similar to that caused by hypertonic solution (Figure 3.5), which suggests that rat MNCs express a Ca^{2+}-dependent PLC pathway that is activated by MNC depolarization and the subsequent Ca^{2+} influx through voltage gated Ca^{2+} channels. The requirement for influx of Ca^{2+} through L-type Ca^{2+} channels is also true for high K^+-induced PLC activation. This effect was blocked by felodipine (30 μM), another L-type Ca^{2+} channel antagonist (Figure 3.5). The high K^+-induced decrease in PIP_2 was also blocked by the presence of a PLC inhibitor (U-73122; 1 μM) or by the absence of added Ca^{2+} in the external solution.

I did not measure the membrane potential of MNCs exposed to the high K^+ solutions (although the Goldman Equation suggests that 30 mM K^+ should depolarize the cells to about -40mV) or the membrane potential of MNCs exposed to +30 mosmol kg^{-1} saline in the presence of TTX, but the results of these experiments imply that AP firing may not be required to initiate the Ca^{2+} influx that activates PLC under the conditions used. It may therefore be relevant that the MNCs express L-type Ca^{2+} channels with a low threshold of activation (Fisher *et al.*, 1995). Whole cell patch clamp studies demonstrated that acutely isolated MNCs express L-type Ca^{2+} currents with a threshold of activation around -50 mV. These low threshold L-type Ca^{2+} currents may be mediated by the $Ca_V1.3$ subtype (Fisher *et al.*, 1995), which has been shown to have a lower threshold of activation than the $Ca_V1.2$ (Lipscombe *et al.*, 2004). Single cell RT-PCR of MNCs has

shown that both $Ca_V1.2$ and $Ca_V1.3$ are expressed in MNCs (Glasgow *et al.*, 1999), and immunological experiments have confirmed that $Ca_V1.3$ is expressed in MNCs (Fisher *et al.*, 2000; Joux *et al.*, 2001). Under the conditions of my experiments, it may be sufficient to depolarize the MNCs enough to open low threshold L-type Ca^{2+} channels that do not require APs to be activated. This does not eliminate the possibility that influx through both subtypes of L-type Ca^{2+} channels (or influx of Ca^{2+} through other channels such as the TRPV1 channels; (Lukacs *et al.*, 2013; Borbiro *et al.*, 2015) may contribute to the activation of PLC *in situ*. Prolonged water deprivation has been shown to increase the expression of L-type Ca^{2+} currents in MNCs (Zhang *et al.*, 2007a) and this could contribute to an increase in the activation of PLC and thereby an enhancement of TRPV1 current when the need for VP release is high.

The activity- and Ca^{2+}-dependence of the osmotic activation of PLC has important implications about the identity of the PLC isoform or isoforms that are responsible. Isoforms of PLC have been grouped into 6 major families (PLC β, PLC γ, PLC δ, PLC ε, PLC ζ, and PLC η) based on their sequence homology and domain structure (Suh *et al.*, 2008b; Kadamur *et al.*, 2013). Different subtypes have been shown to be activated by different types of stimuli, including activation of G-protein coupled receptors (GPCRs) and increases in intracellular Ca^{2+} (Rhee, 2001; Suh *et al.*, 2008b). The PLC β isoforms, for example, are activated by GPCRs, whereas PLC δ isoforms are Ca^{2+}-dependent. Four different PLC isoforms (PLC β4, PLC δ1, PLC δ4 and PLC γ1) have been detected in the SON using DNA microarrays (Hazell *et al.*, 2012). MNCs also express GPCRs such as Ang II (Zhang *et al.*, 2008; Hazell *et al.*, 2012) and muscarinic acetylcholine receptors (Ghamari-Langroudi *et al.*, 2004; Hazell *et al.*, 2012; Shah *et al.*, 2014) that are linked to the activation of PLC (Rhee, 2001; Suh *et al.*, 2008b). It is unlikely however that GPCR activation could be involved in the osmotic activation of acutely isolated MNCs, because any released

signaling molecules would rapidly diffuse into the large volume of the bathing solution. The Ca^{2+} dependence of the osmotic activation of PLC suggests the possibility that while the PLC β4 isoform is responsible for the GPCR-dependent activation of PLC, one or both of the PLC δ isoforms is responsible for the osmotic activation of PLC. Further studies will be required to identify the PLC isoforms underlying these responses.

Ang II has been shown to enhance osmosensory transduction in MNCs through a mechanism that depends on activation of PLC and PKC (Zhang *et al.*, 2008). I therefore hypothesized that PLC may contribute to the osmotic activation of TRPV1 currents. I measured ramp currents in acutely isolated rat MNCs using whole cell patch clamp before and after increases in osmolality in the presence and absence of the PLC inhibitor U73122 (1 μM). The exposure to increased external osmolality increased a current whose properties are consistent with those of the osmosensitive TRPV1-mediated current (Oliet *et al.*, 1993a; Sharif Naeini *et al.*, 2006). In the presence of the PLC inhibitor this response was diminished (Figure 3.6), which supports the hypothesis that the osmotic activation of PLC contributes to the activation of this current.

The activation of PLC leads to a decrease in PIP_2 and an increase in IP_3 and DAG and I next wanted to test which of these effects mediates the enhancement of the TRPV1 current. I tested whether a decrease in PIP_2 was responsible by repeating measurement of the ramp currents before and after administration hypertonic solution with and without the inclusion of the PIP_2 analogue PIP_2-diC8 (100 μM) in the patch pipette. I predicted that if the osmotic activation of PLC was enhancing the activation of TRPV1 currents by decreasing PIP_2, the enhancement should be prevented by the presence of the PIP_2 analogue and thus the current evoked by the increase in

osmolality should be smaller. I found that the response to increased osmolality was not decreased by including the PIP_2 analogue in the patch pipette (Figure 3.7), which suggests that the PLC-mediated enhancement of osmotically-evoked TRPV1 currents does not depend on a decrease in PIP_2.

I next tested whether the PLC-dependent enhancement of osmotically-evoked TRPV1 currents depends on the activation of PKC. I show in Figure 3.8 that the PKC inhibitor GF109203X (2 μM) is able to suppress the osmotic activation of the TRPV1 current whereas an inactive analogue was not (Figure 3.8). This suggests that the PLC-mediated enhancement of the osmotic response depends on the activation of PKC. Lastly, I tested whether direct activation of PKC would enhance TRPV1-mediated currents in the absence of a change in external osmolality. In Figure 3.9 I show that treatment with a PKC activator does indeed increase a current with properties consistent with those expected for the TRPV1-mediated current. Furthermore, treatment with this PKC activator enhances the activation of TRPV1 currents caused by exposure to hypertonic saline. These data support a role for the activation of PLC in the osmotic activation of TRPV1 currents.

Ang II has been shown to enhance osmosensory transduction in MNCs through a mechanism that depends on activation of PLC and PKC (Zhang *et al.*, 2008). My data are consistent with these results and suggest that the effects of osmotic stimulation overlap with those of Ang II because both are causing an activation of PLC and PKC. While the PKC-mediated effect of Ang II on osmotic transduction depends on an enhancement of cortical actin density (Zhang *et al.*, 2008), work in other cell types has shown that PKC can enhance the activity of TRPV1 channels by phosphorylating them at specific sites (Numazaki *et al.*, 2002; Plant *et al.*,

2007). I have not tested whether the osmotic activation of PLC and PKC enhances TRPV1 currents through actions on the cortical actin network or by a direct phosphorylation of TRPV1 channels.

MNCs transduce osmotic signals into electrical firing patterns that regulate the body fluid homeostasis by varying the secretion of VP and OT. This transduction depends on the osmotic activation of mechanosensitive TRPV1 channels in the MNCs (Sharif Naeini *et al.*, 2006; Zaelzer *et al.*, 2015) and in other osmosensitive neurons in neighbouring brain regions (Ciura *et al.*, 2006; Ciura *et al.*, 2011). Myr results show that the osmotic activation of PLC, through a Ca^{2+}-dependent mechanism, may also contribute to the osmosensitivity of MNC by enhancing activation of the TRPV1 currents in rat MNCs. I show that this effect is mediated by PKC and is not due to decreased membrane PIP_2 levels. It remains to be seen whether this pathway contributes to the function of other osmosensitive neurons. My work suggests that osmotic activation of PLC might be an important contributor to MNC osmosensitivity and to osmoregulation of body fluid balance.

APPENDIX 3A

THE ROLE OF PLC δ1 IN MEDIATING THE OSMOTICALLY-EVOKED PIP_2 DECREASE IN ACUTELY ISOLATED MOUSE MNCs

Although four different PLC isoforms that includes PLC β4, PLC δ1, PLC δ4 and PLC γ1 have been previously detected in SON by DNA microarrays (Hazell *et al.*, 2012), the physiological roles of these isoforms in MNC physiology have not been studied. Since PLC δ1 and PLC δ4 are highly Ca^{2+} sensitive and have been shown to activate in response to intracellular Ca^{2+} increases (Allen *et al.*, 1997; Kim *et al.*, 1999), my data suggests that either the PLC δ1 and/or the PLC δ4 isoform could be the Ca^{2+}-dependent PLC isoform that is osmotically-activated in the MNCs. To ascertain the role of PLC δ1 isoform, we therefore collaborated and obtained PLC δ1 knockout mice (Ichinohe *et al.*, 2007; Nakamura *et al.*, 2008) from Dr. K. Fukami (Laboratory of Genome and Biosignals at Tokyo University of Pharmacy and Life Sciences, Japan).

To study the osmotically evoked PIP_2 response in mice MNC, I first standardized the PIP_2 immunocytochemistry model in acutely isolated wild type mouse MNCs. Briefly, the composition of previously used isotonic saline was slightly modified to raise its osmolarity to 310 mosmol kg^{-1} to match it to the iso-osmotic set point of mice (Bourque *et al.*, 2007; Bourque, 2008). Thereafter 340 mosmol kg^{-1} hypertonic saline was prepared by adding mannitol to this isotonic saline solution as was done for rat MNCs.

The normalized PIP_2 immunoreactivity observed in wild type mouse MNCs during these experimental conditions were as follows: isotonic 310 mosmol kg^{-1} saline (control; 100.0 ± 7; n = 7 experiments) and 340 mosmol kg^{-1} hypertonic saline (83.9 ± 7.8; n = 7 experiments). Whereas, the normalized PIP_2 immunoreactivity observed in PLC δ1 knock out mouse MNCs were as follows:

isotonic 310 mosmol kg^{-1} saline (control; 100.0 ± 5.2; *n* = 5 experiments), 340 mosmol kg^{-1} hypertonic saline (77.2 ± 6.7; *n* = 5 experiments). The results of these experiments are illustrated in Figure 3.10. Although the wild type MNCs responded to the 5 minute hypertonic stimulation with a nearly 20% decrease in their membrane PIP_2 levels much like the rat MNCs, this response was not statistically different from the one that was observed in MNCs obtained from PLC δ1 knockout mice. This suggests that PLC δ1 isoform might not contribute to the osmotically-evoked PLC effects in MNCs and therefore further experiments to test role of PLC δ4 needs to be performed.

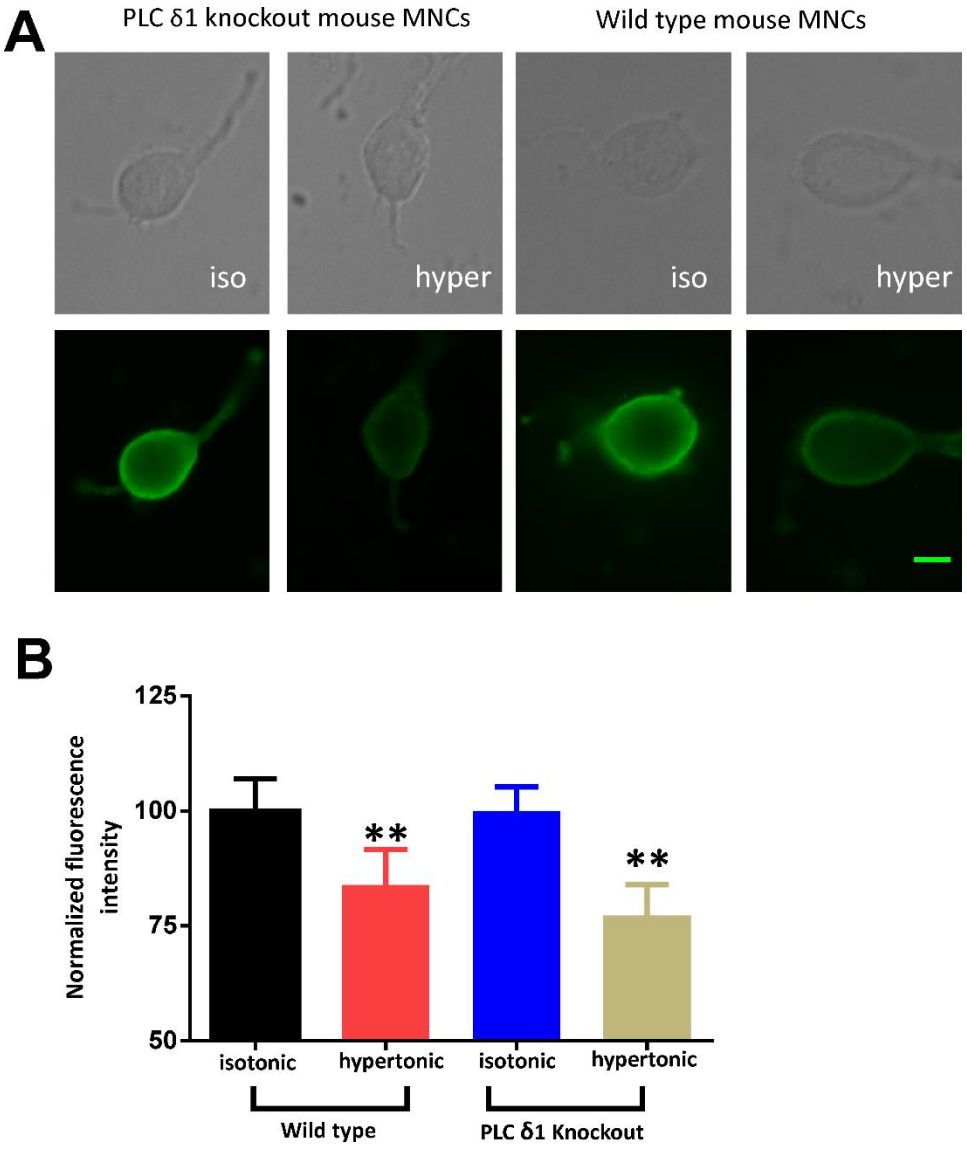

Figure 3.10. Exposure to hypertonic saline causes a decrease in immunoreactivity to PIP_2 in the plasma membrane of isolated MNCs obtained from both wild type and PLC δ1 knockout mice. A, Representative images of isolated mouse MNCs using either differential interference contrast (upper panels) or fluorescence microscopy showing immunoreactivity for PIP_2 (lower panels). Cells were maintained in isotonic 310 mosmol kg^{-1} saline (control), or exposed to hypertonic 340 mosmol kg^{-1} saline (hypertonic) for 5 minutes each. The scale bar is 20µm. B, Bar graphs showing the normalized immunoreactivity to PIP_2 in MNCs in different treatment groups. Data is expressed as mean ± SEM (** $p<0.01$).

PREFACE TO CHAPTER 4:

The following chapter is a published paper that was published in Journal of Neuroendocrinology in year 2016. The full citation of this paper is as follows;

Na^+-activated K^+ channels in rat supraoptic neurons. J Neuroendocrinol. 2016 Jun; 28(6).

Bansal V and Fisher TE.

Being the first author of this paper, I have made some minor modifications like changing the page numbers and figure numbers to make it fit in my manuscript based thesis format. My experimental contributions in this paper includes doing all electrophysiology and immunostaining experiments on rat MNCs. I am responsible for all the experimental data that was shown in Figures 1-5 of this paper.

In this paper, I have reported the novel evidence that demonstrated the presence of functional sodium activated potassium channels (K_{Na}) in rat MNCs. Specifically I have shown that a majority of rat MNCs have an outwardly rectifying K^+ current that is dependent on Na^+ influx mediated by TTX sensitive Na^+ channels. I have also reported immunocytochemical evidence for the presence of Slack (slo 2.2) and Slick (slo2.1) proteins in rat MNCs, which are reportedly the structural proteins for forming K_{Na} channels.

CHAPTER 4

NA^+-ACTIVATED K^+ CHANNELS IN RAT SUPRAOPTIC NEURONS

4.1 ABSTRACT

The magnocellular neurosecretory cells (MNCs) of the hypothalamus secrete the neurohormones vasopressin and oxytocin. The systemic release of these hormones depends on the rate and pattern of MNC firing and it is therefore important to identify the ion channels that contribute to the electrical behaviour of MNCs. In this study, I present evidence for the presence of Na^+-activated K^+ (K_{Na}) channels in rat MNCs. K_{Na} channels mediate outwardly rectifying K^+ currents that are activated by the increases in intracellular Na^+ that occur during electrical activity. Although the molecular identity of native K_{Na} channels is unclear, their biophysical properties are consistent with those of expressed Slick (slo 2.1) and Slack (slo 2.2) proteins. Using immunocytochemistry and Western blot experiments, I found that both Slick and Slack proteins are expressed in rat MNCs. Using whole cell voltage clamp techniques on acutely isolated rat MNCs, I found that inhibiting Na^+ influx by the addition of the Na^+ channel blocker tetrodotoxin (TTX) or the replacement of Na^+ in the external solution with Li^+ caused a significant decrease in sustained outward currents. Furthermore, the evoked outward current density was significantly higher in rat MNCs using patch pipettes containing 60 mM Na^+ than it was when patch pipettes containing 0 mM Na^+ were used. My data shows that functional K_{Na} channels are expressed in rat MNCs. These channels could contribute to the activity-dependent afterhyperpolarizations that have been identified in the MNCs and thereby play a role in the regulation of their electrical behaviour.

4.2 INTRODUCTION

The magnocellular neurosecretory cells (MNCs) of the supraoptic (SON) and paraventricular nuclei (PVN) of the hypothalamus are responsible for the synthesis and release of the neurohormones vasopressin (VP) and oxytocin (OT). MNCs typically release either VP, which acts on the kidneys to control water excretion (Nielsen *et al.*, 1995) and is the major osmoregulatory hormone, or OT, which plays an important role in lactation and parturition, but also has a role in osmoregulation by causing natiuresis (Conrad *et al.*, 1993) at least in some species. Both types of MNCs respond to increases in plasma osmolality by increasing their systemic release of VP and OT. Both VP and OT MNCs tend to fire slowly and irregularly when plasma osmolality is near the normal physiological set point (which is 295 mosmol kg^{-1} in rats), but fire more frequently as the external osmolality increases (Poulain *et al.*, 1982; Bourque *et al.*, 1997). This osmotically-evoked increase in firing is due to the intrinsic osmosensitivity of MNCs, which is caused by mechanosensitive activation of stretch-inactivated channels (Oliet *et al.*, 1993a) mediated by a trpv1 channel variant (Sharif Naeini *et al.*, 2006; Zaelzer *et al.*, 2015), and by increased excitation from osmosensitive neurons in nearby hypothalamic nuclei (Richard *et al.*, 1995). The release of VP and OT is elevated as the rate of MNC firing increases and is further enhanced by burst firing (Bicknell, 1988). VP MNCs undergo an osmotically-evoked transition to phasic burst firing (characterized by bursts of APs lasting tens of seconds) that maximizes release, while OT MNCs show maximal release during high frequency bursts that occur during lactation and parturition. The transition to burst firing is thought to involve a variety of activity-dependent currents, including Ca^{+} -activated K^{+} (K_{Ca}) currents. The Ca^{2+} influx that occurs during APs activates K_{Ca} currents leading to afterhyperpolarizations (AHPs) of different kinetics. K_{Ca} currents contribute to the fast AHP (fAHP) that is activated by a single AP and to the slow AHP (sAHP) and medium AHP (mAHP) that are

relatively slow to activate and are typically seen after multiple APs. All three of these AHPs are important in regulating the firing patterns of MNCs (Kirkpatrick *et al.*, 1996; Dopico *et al.*, 1999; Ghamari-Langroudi *et al.*, 2004; Greffrath *et al.*, 2004; Ohbuchi *et al.*, 2010) and other neurons (Sah *et al.*, 2002; Faber *et al.*, 2003). An increase in Na^+ influx during APs has been shown in other neurons to activate Na^+-activated K^+ (K_{Na}) channels, which also mediate outwardly rectifying K^+ currents and thus contribute to the generation of fAHP (Liu *et al.*, 2004; Gao *et al.*, 2008) and sAHPs (Sanchez-Vives *et al.*, 2000; Wallén *et al.*, 2007). It is not known whether K_{Na} channels are expressed in MNCs or whether they contribute to the generation of AHPs and to the regulation of MNC electrical behaviour.

The molecular identity of native K_{Na} channels is still incompletely understood, but similar Na^+-activated K^+ currents have been observed following the expression of two proteins commonly called Slick (Slo 2.1) and Slack (Slo 2.2), which have homology with Ca^{2+}-activated K^+ channels (Bhattacharjee *et al.*, 2005a). Both Slack and Slick channels are voltage-dependent and have been shown to mediate outwardly rectifying K^+ currents at potentials above -50 mV (Bhattacharjee *et al.*, 2003; Yuan *et al.*, 2003). The Na^+ dependence of Slack and Slick was first demonstrated by increasing the Na^+ levels on the cytoplasmic face of excised patches containing expressed Slack and Slick channels, which caused an increase in their opening probability (Bhattacharjee *et al.*, 2003). Slack and Slick are widely expressed in rat brain (Bhattacharjee *et al.*, 2002; Bhattacharjee *et al.*, 2005b) and K_{Na} channels may be important in regulating the electrical behaviour of a wide variety of neurons (Kim *et al.*, 2014).

The study of K_{Na} channels is made difficult by lack of specific pharmacological antagonists (Bhattacharjee *et al.*, 2005a). The most common methods employed to test the presence of functional K_{Na} channels in cells are therefore based on manipulating intracellular Na^+ levels or Na^+

influx. The blockade of Na^+ influx through tetrodotoxin (TTX) sensitive Na^+ channels has been used to demonstrate K_{Na} currents in vestibular (Cervantes *et al.*, 2013) and olfactory neurons (Lu *et al.*, 2010). Substitution of external NaCl with LiCl has also been used to reveal the presence of Na^+-activated K^+ currents in neurons (Hess *et al.*, 2007; Cervantes *et al.*, 2013), because Li^+ permeates Na^+ channels (Hille, 1972) without activating K_{Na} currents (Dryer *et al.*, 1989; Bischoff *et al.*, 1998). Similarly, manipulations of internal Na^+ concentrations in patch clamp experiments have been used to demonstrate the presence of K_{Na} currents in neurons (Yang *et al.*, 2007; Lu *et al.*, 2010).

Immunohistochemical evidence suggests that Slick is expressed in the SON (Bhattacharjee *et al.*, 2005b) but the cell type or types that express Slick were not identified. Work by the same laboratory suggested that the Slack-B isoform is not expressed in the SON (Bhattacharjee *et al.*, 2002), although it remains possible that other Slack isoforms might be expressed by the MNCs. I therefore tested whether Slick and other Slack isoforms are expressed in the MNCs using immunocytochemical techniques and whether rat MNCs display K_{Na} currents using electrophysiological techniques. I report that rat MNCs express both Slick and Slack and display K_{Na} currents. These currents could contribute to the regulation of MNC firing patterns and therefore could play an important role in regulating the release of VP and OT.

4.3 MATERIALS AND METHODS

4.3.1 Ethical approval

This work was approved by the University of Saskatchewan's Animal Research Ethics Board and adhered to the Canadian Council on Animal Care guidelines for humane animal use.

4.3.2 Chemicals

All drugs and chemicals used in the following experiments were purchased from Sigma unless stated otherwise.

4.3.3 Animals and cell preparation

MNCs were isolated using a protocol described previously (Liu *et al.*, 2005) and were identified as those cells having a cross-sectional area greater than 160 μm^2 (Oliet *et al.*, 1992). I also used immunocytochemical techniques to identify cells expressing either VP or OT (see below). In brief, male Long–Evans rats (200–300 g) were anaesthetized with isoflurane and killed by decapitation on the day of the experiment. The brain was rapidly removed and blocks of tissue containing most of the two supraoptic nuclei were carefully excised. The tissue blocks were incubated with an oxygenated (100% O_2) Pipes solution (pH 7.1) composed of (in mM): 120 NaCl, 5 KCl, 1 $MgCl_2$, 1 $CaCl_2$, 20 Pipes, 10 glucose and trypsin (Type XI, 0.6 mg ml^{-1}) for 90 min at 34°C. After enzymatic treatment, the SON blocks were transferred into oxygenated Pipes solution (pH 7.4) without trypsin for 30 min at room temperature. The osmolality of Pipes was adjusted to 295 ± 3 mosmol kg^{-1} using a VAPRO pressure osmometer (WESCOR; Logan, UT, USA) by adding mannitol as required. Finally, the tissue were gently triturated with fire-polished pipettes to disperse the MNCs onto glass-bottomed culture dishes, which were kept at room temperature for electrophysiological or immunocytochemical experiments as described below.

4.3.4 Immunocytochemical methods

Standard immunocytochemistry methods were used to test for the presence of Slack and Slick proteins in rat MNCs. Briefly, MNCs acutely isolated from four rats were plated on glass bottom dishes and fixed with phosphate-buffered saline (PBS) containing 4% paraformaldehyde for 20–25 min at room temperature. Following three washes with PBS, the cells were blocked with solution containing 10% donkey serum and 0.02% Triton X for 1 h. The cells were then incubated with the primary antibodies overnight at 4°C. The following commercial primary antibodies were used: mouse monoclonal Slack antibody (clone N3/26, dilution 1:50; NeuroMab, Davis, CA, USA) and

mouse monoclonal Slick antibody (clone N11/33, dilution 1:50, NeuroMab). I also used antibodies against neurophysin II (which is expressed only in VP MNCs) and neurophysin I (which is expressed only in OT MNCs) in dual labeling studies to determine whether both types of MNCs express Slick and Slack. These antibodies (Altstein *et al.*, 1988) - rabbit polyclonal vasopressin (VA 4, dilution 1:1000) and rabbit polyclonal oxytocin (VA 10, dilution 1:1000)- were received as a gift from Dr Harold Gainer (NIH, Bethesda, USA). Negative controls were also included by omitting incubation with the primary antibodies in one subset of MNCs for each experiment. On the next day, the dishes were washed with PBS three times and incubated for another 1 h with secondary antibodies. After three washes with PBS, Citifluor mounting solution (Citifluor Ltd; Gore, QC, Canada) was added to the dishes and cells were then viewed using a Zeiss inverted Axiovert 200 microscope with appropriate filter sets and a ×40 objective. Images were captured using a cooled CCD camera. The images were processed and analysed with Image J software (NIH). I defined MNCs as either VP or OT MNC if they showed immunofluorescence for their respective neurophysin that was equal to or greater than four times the mean immunofluorescence for the negative control MNCs on each experimental day. MNCs were considered to be positive for Slack and Slick if the immunofluorescence for the appropriate antibody was equal to or greater than three times the mean immunofluorescence for their negative control MNCs.

4.3.5 Western blot methods

SON blocks were rapidly removed from the brains of four decapitated adult male Long-Evans rats and homogenized and the proteins were solubilized and separated using standard electrophoresis techniques. Care was taken to ensure that the excised blocks contained the greatest possible percentage of SON tissue and the least possible of non-SON tissue. I also used samples from whole rat brain, whole mouse brain, rat heart, and rat liver as control tissues. All procedures

were performed on ice to prevent proteolysis of Slack and Slick subunits. The homogenization buffer contained 50 mM Tris (pH 7.4), 0.3 M sucrose, 0.1 M NaCl, one tablet of Complete Mini (Roche Diagnostics, Indianapolis, IN, USA), 100 µg/ml benzamidine, 1 µg/ml pepstatin and 10 µg/ml calpain inhibitor. The tissue lysates were centrifuged for 15 min at 35000 g, and the supernatants were collected for immunoblot analysis. After separation by SDS-PAGE, proteins were transferred from the gels to nitrocellulose membranes (Amersham Biosciences Corporation, Little Chalfont, UK). The membranes were then blocked by 5% non-fat milk in PBS-Tween for 1 h, followed by overnight incubation of Slack and Slick antibodies (dilution 1: 500; NeuroMab) at 4°C in the blocking solution. The next day the membranes were washed with PBS-Tween and incubated with horseradish peroxidase-coupled anti-mouse (Bio-Rad Laboratories) secondary antibodies for 1 hour. The membranes were then washed with PBS-Tween and treated with ECL Advance Western Blotting Detection Kits (Amersham Biosciences Corporation) according to the manufacturer's protocol. X-ray films were exposed and developed according to standard protocols and the resulting bands were scanned and analyzed using Image J software.

4.3.6 Electrophysiological methods

MNCs acutely isolated from seventeen rats were perfused with an external recording solution containing (in mM) 140 NaCl, 5 KCl, 10 Hepes, 1 $MgCl_2$, 0.1 $CdCl_2$, 0.2 NiCl, 10 glucose, and 0.01 glybenclamide (pH 7.4) and placed on the microscope stage of the electrophysiology station for current measurements. The Ca^{2+} was omitted and Cd^{2+} and Ni^{2+} were included to inhibit the Ca^{2+} currents and the participation of K_{Ca} channels in my recordings while glybenclamide was used to inhibit ATP-sensitive K^+ channels. The osmolalities of this and other all external recording solutions, as discussed below, were adjusted to 295 ± 3 mosmol kg^{-1} using mannitol. MNCs were subjected to whole cell patch clamp using patch pipettes having resistances of 2-4 MΩ and filled

with an internal solution containing (in mM) 130 KCl, 10 Hepes, 1 $MgCl_2$, and 5 EGTA (pH 7.2), unless stated otherwise. Borosilicate glass capillaries (A-M Systems, Carlsborg, WA, USA) were used to pull patch pipettes on a P-97 horizontal pipette puller (Sutter Instrument Company, Novato, USA) and fire-polished using a microforge (Narashige, Tokyo, Japan). The macroscopic whole cell currents in rat MNCs were evoked and recorded using an EPC-9 amplifier (HEKA Elektronik, Lambrecht/Pfalz, Germany) controlled with PULSE software (HEKA). The signals were low-pass filtered at 2 kHz and digitized at 20 kHz for all voltage clamp experiments. The cells were held at -70 mV and the currents evoked in response to voltage clamp protocols as described in the text. Currents evoked after a treatment were digitally subtracted from the control currents to show the effect of that treatment. All current measurements were made using PULSEFIT software (HEKA) and where indicated were divided by whole cell capacitance values to determine current density (expressed as pA/pF). The current density values were pooled and expressed as mean ± SEM for each experimental group and statistically compared with the control group values at the same potential using the Student's paired t test. The differences were deemed significant if $P < 0.05$. I have not included data from MNCs that did not show the effect of different treatments on the evoked K^+ currents to give a better indication of the Na^+ dependence of K^+ currents in my experiments. The inclusion of the non-responding MNCs did not change the level of significance in statistical comparisons made in any treatment group. The normalized conductance-voltage curves (G/G_{max} vs. test potentials) were plotted using the Boltzmann sigmoidal fit equation using Graph Pad software to estimate the half-activation potential ($V_{1/2}$) of Na^+-activated K^+ currents in different experiments along with the slope of the Boltzmann fit.

4.4 RESULTS

4.4.1 Immunostaining experiments

Immunohistochemical results suggest that Slick is expressed in the SON, but the cell type or types that express Slick were not identified (Bhattacharjee *et al.*, 2005b). Moreover, the researchers who reported no immunoreactivity for Slack in the SON (Bhattacharjee *et al.*, 2002) used an antibody that was later found to recognize an epitope present in only one Slack isoform, which is now called Slack-B (Brown *et al.*, 2008). Five mRNA transcripts (Slack-A, Slack-Ax2, Slack-B, Slack-Bx2 and Slack-M) have been identified in the mouse brain (Brown *et al.*, 2008), which raises the possibility that Slack isoforms other than Slack-B could be present in rat MNCs.

I therefore employed immunostaining methods to probe for Slick and Slack proteins in acutely isolated rat MNCs and used dual-labelling techniques to identify VP and OT MNCs. I employed primary antibodies that have been used to demonstrate the presence of Slack and Slick in other neurons (Cervantes *et al.*, 2013). The Slack antibody that I chose targets a C-terminal sequence that may be present in all Slack isoforms (Brown *et al.*, 2008). I found that 25% (20/79) of the MNCs isolated in my test dishes were positive for neurophysin I (i.e. were OT MNCs). Almost all of the OT MNCs tested were immunopositive for Slick (8/9) and for Slack (11/11), suggesting that most or all OT MNCs express both proteins (Figure 4.1A). Eighty two percent (80/97) of the MNCs in my test dishes were positive for neurophysin II (i.e. were VP MNCs). Almost all of the tested VP MNCs were positive for both Slick (40/41) and Slack (37/39; Figure 4.1A). These data suggest that Slack and Slick are co-expressed in both VP and OT MNCs and may contribute to the formation of K_{Na} channels in MNCs. The lack of immunoreactivity in the SON for an antibody directed towards Slack-B (Bhattacharjee *et al.*, 2002) suggests that the MNCs might express an isoform of Slack other than Slack-B.

4.4.2 Western blot experiments

I also used the same antibodies to probe immunoblots prepared from mouse brain, rat brain, and rat SON homogenates. Clear bands close in size to the expected molecular weight for Slack and Slick proteins (between 120-140 kDa; (Chen *et al.*, 2009)) were detected in all three of these lanes (Figure 4.1B). As expected, no bands were seen in lanes of rat liver homogenates (Bhattacharjee *et al.*, 2003; Yuan *et al.*, 2003), while a weak signal for the presence of Slick protein (at about 100 kDa) was seen in the lane of rat heart homogenate in some of my experiments (data not shown).

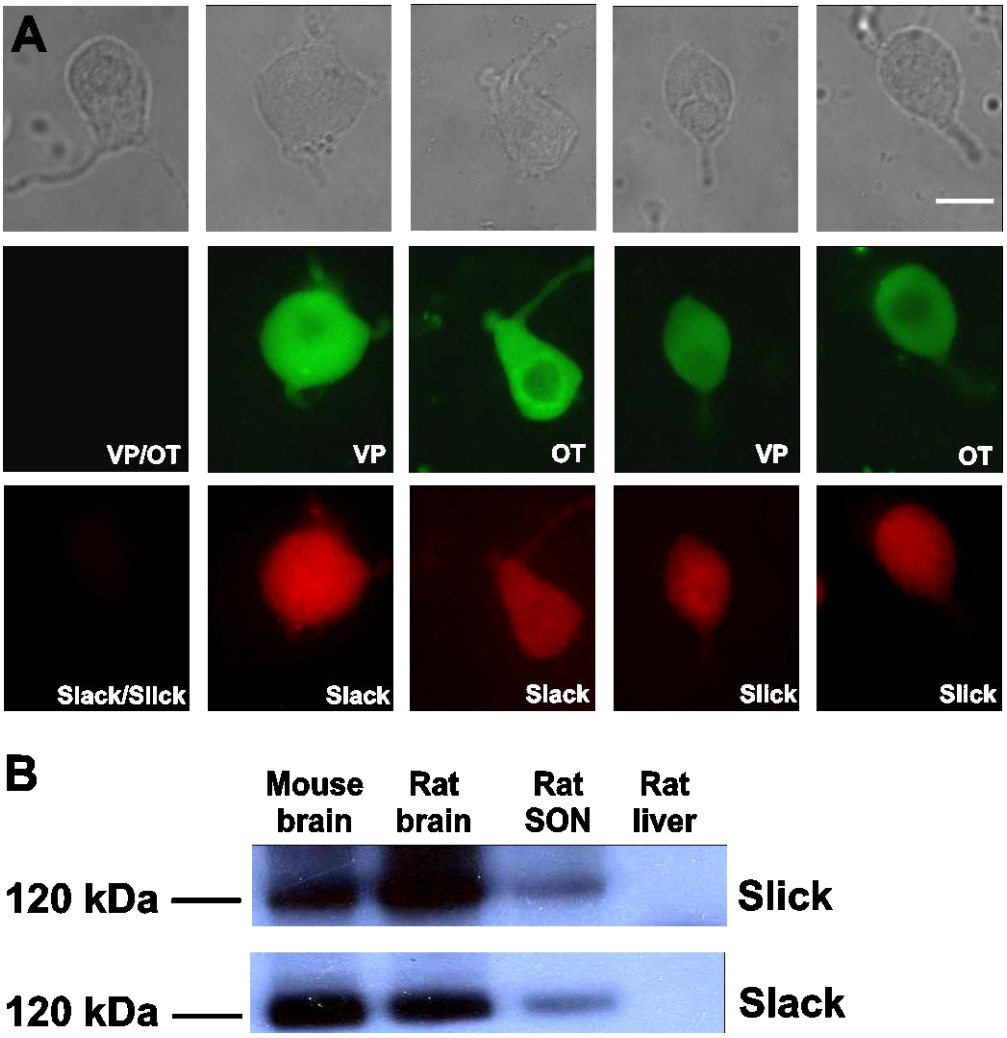

Figure 4.1. Immunoreactivity for Slack (slo 2.2) and Slick (slo 2.1) proteins in rat MNCs.
A, Phenotypically-identified rat VP and OT MNCs were probed for the presence of Slack or Slick proteins using dual immunolabeling. Representative differential interference contrast images (top panels) and fluorescence images of MNCs probed for VP and OT (in green; middle panels) and Slack and Slick (in red; bottom panels) are shown. The presence of Slack and Slick proteins was detected in both VP and OT MNCs, while no signal was detected in the negative control group. The scale bar indicates 20 µm. B, Immunoblots indicate the presence of Slack and Slick proteins in rat SON homogenates. The immunoblots of homogenates prepared from rat SON and control tissues (mouse brain, rat brain, and rat liver) were probed with same set of slack and slick antibodies that were used in immunocytochemistry experiments. A clear band was detected close to the expected molecular weight of slightly above 120 kDa for both slack and slick proteins in the rat SON and in the positive control tissues (rat brain and mouse brain) but not in the negative control tissue (rat liver).

4.4.3 TTX inhibits outward currents

I used whole cell voltage clamp techniques to test for the presence of K_{Na} channels in acutely isolated rat MNCs. The cell potential was stepped from a holding potential (V_H) of -70 mV to a series of test potentials between -40 to +40 mV in 20 mV increments for 100 ms every 5 seconds. This protocol resulted in generation of fast-activating inward currents that were followed by relatively slowly activating outward currents. After recording the control currents, 1 µM TTX (a Na^+ channel blocker) was bath applied and the currents were re-recorded using the same protocol. The digital subtraction of the currents measured before and after TTX application revealed a typical TTX-sensitive fast inward current (which peaked in < 2 ms) that was followed by a TTX-sensitive slowly activating (reaching peak current at from 5 to 20 ms) and sustained (< 5% inactivation in 100 ms) outward current in about 60% (8 out of 14) of the MNCs tested (Figure 4.2A). The observation of this TTX-sensitive outward current in rat MNCs is consistent with the presence of K_{Na} channels as reported in vestibular neurons (Cervantes *et al.*, 2013). The mean outward currents recorded in the responding cells (n=8) before and after TTX treatment were normalized and plotted to show the current density at all tested potentials (Figure 4.2B). The effect of 1 µM TTX on outward current density was statistically significant at potentials of 0 mV and above. The mean outward current density recorded before and after 1 µM TTX addition at -40 mV was 19 ± 5 pA/pF and 19 ± 5 pA/pF ($p > 0.05$), at -20 mV was 65 ± 9 pA/pF and 63 ± 8 pA/pF ($p > 0.05$), at 0 mV was 141 ± 13 pA/pF and 124 ± 12 pA/pF ($p < 0.05$), at 20 mV was 245 ± 18 pA/pF and 207 ± 16 pA/pF ($p < 0.05$) and at 40 mV was 362 ± 29 pA/pF and 309 ± 27 pA/pF ($p < 0.05$). The voltage for half activation ($V_{1/2}$) of the TTX-sensitive outward current was found to be -4.0 ± 4.8 mV when plotted using the Boltzmann fit equation. The slope of this fit was 12.5 ± 4.4 (Figure 4.2C).

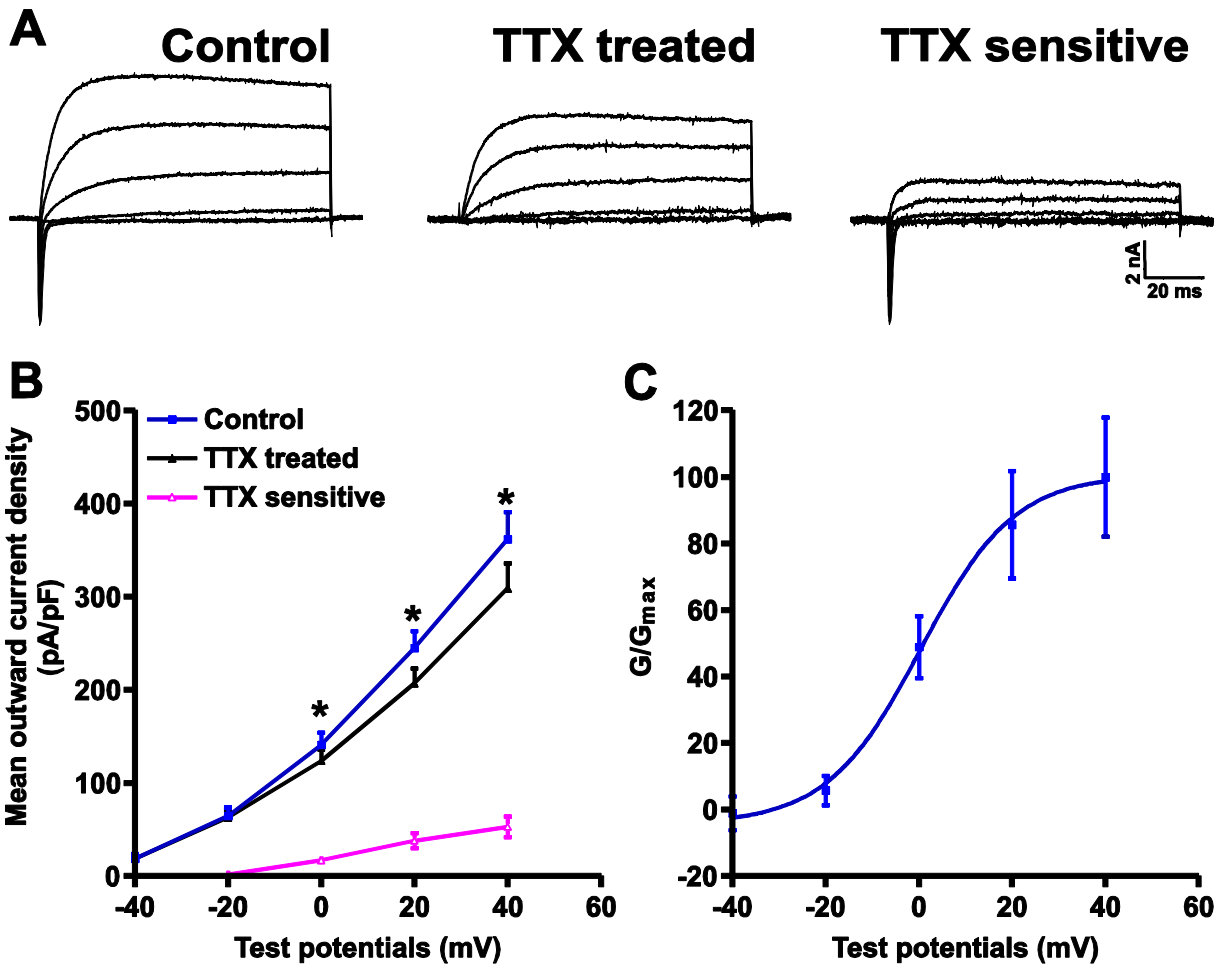

Figure 4.2. TTX inhibits outward currents in acutely isolated rat MNCs.
A, Whole-cell recordings showing representative currents evoked by stepping from −40 to +40 mV in 20 mV increments every five seconds from a holding potential of -70 mV. The internal and external solutions used in these experiments are described in the Materials and Methods section. 'Control' refers to currents evoked in an MNC before and 'TTX treated' refers to currents evoked in the same MNC after the application of 1 µM TTX to the bathing solution. The digitally-subtracted currents show the 'TTX-sensitive' currents. B, The mean current density of outward currents under control and TTX treated conditions are shown, along with the TTX-sensitive component, as a function of test potentials used. The current density values in the TTX treated group are statistically lower than control values at all potentials at and above 0 mV ($P < 0.05$; indicated by an asterisk) . C, The normalized conductance–voltage curve for the TTX-sensitive outward current was plotted using the Boltzmann fit. The $V_{1/2}$ and slope value of the curve are - 4.0 ± 4.8 mV and 12.5 ± 4.4 respectively.

4.4.4 LiCl substitution inhibits outward currents

I measured currents before and after iso-osmotic substitution of NaCl in the external solution with LiCl (Cervantes *et al.*, 2013; Markham *et al.*, 2013)as a further test for the presence of K_{Na} currents. The digital subtraction of the currents evoked before and after LiCl substitution revealed a Na^+-sensitive outward current similar to the TTX-sensitive outward current described above in about 60% (7 out of 11) of the MNCs tested (Figure 4.3A). The mean outward currents recorded in the responding cells (n=7) before and after LiCl substitution were then normalized and plotted (Figure 4.3B). The effect of LiCl substitution on outward current density was found to be statistically significant at potentials of 0 mV and above. The mean outward current density recorded before and after LiCl substitution at -40 mV was 24 ± 5 pA/pF and 24 ± 5 pA/pF ($p > 0.05$), at -20 mV was 72 ± 6 pA/pF and 71 ± 5 pA/pF ($p > 0.05$), at 0 mV was 127 ± 11 pA/pF and 112 ± 9 pA/pF ($p < 0.05$), at 20 mV was 229 ± 23 pA/pF and 201 ± 21 pA/pF ($p < 0.05$), and at 40 mV was 332 ± 32 pA/pF and 295 ± 29 pA/pF ($p < 0.05$). The voltage for half activation ($V_{1/2}$) of the Na^+ sensitive outward current was found to be -2.3 ± 1.4 mV when plotted using the Boltzmann fit equation. The slope of this fit was 8.7 ± 1.1 (Figure 4.3C).

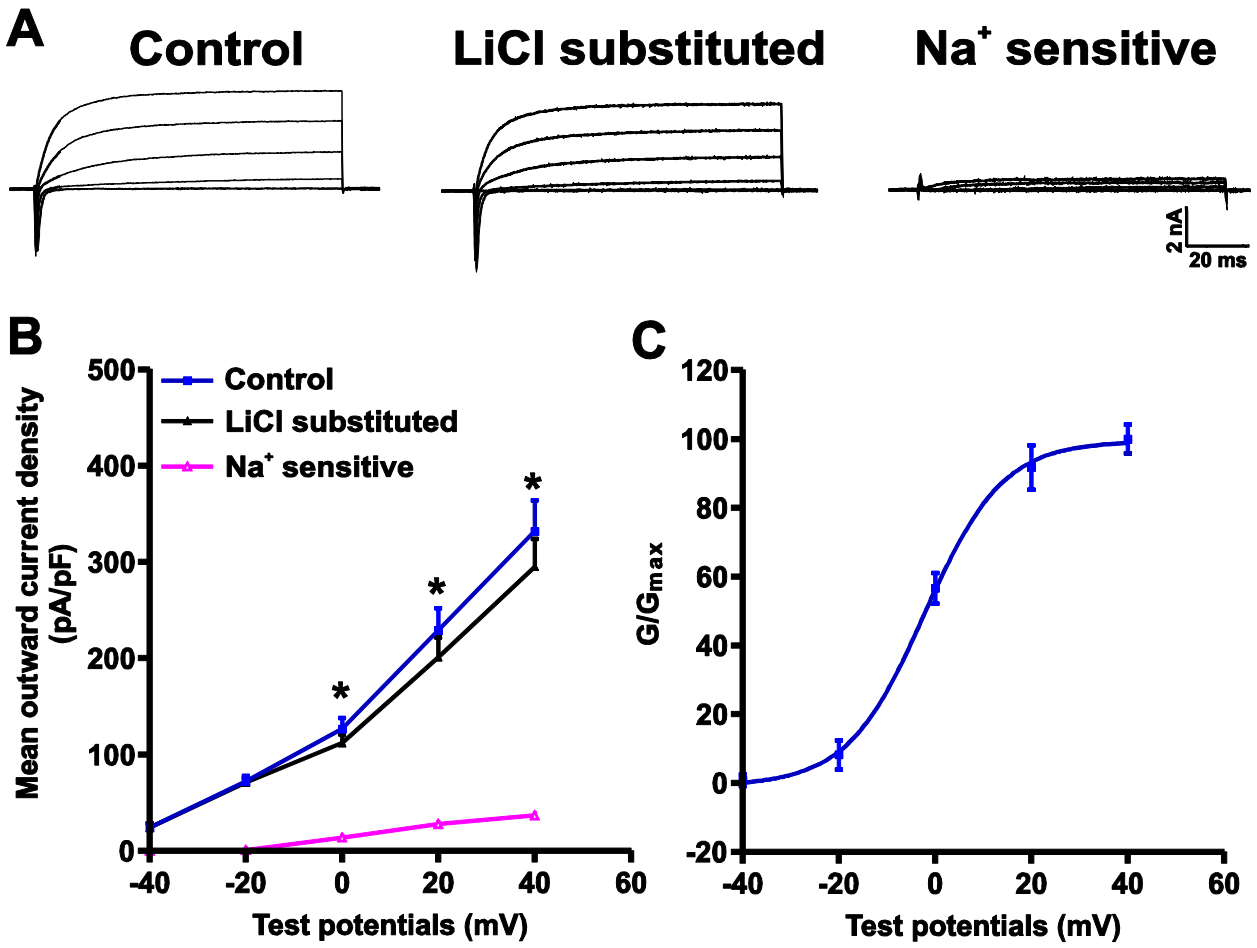

Figure 4.3. LiCl substitution inhibits outward currents in acutely isolated rat MNCs.
A, Whole-cell recordings showing representative currents evoked by stepping from −40 to +40 mV in 20 mV increments every five seconds from a holding potential of -70 mV. The internal and external solutions used in these experiments are described in the Materials and Methods section. 'Control' refers to currents evoked in an MNC bathed in normal external solution and 'LiCl substituted' refers to currents evoked in the same MNC after replacing the external solution with another in which NaCl has been iso-osmotically substituted with LiCl. The digitally-subtracted currents show the 'Na^+ sensitive' currents for the two conditions. *B*, Mean current density of outward currents under control and LiCl substituted conditions are shown, along with the LiCl sensitive component, as a function of test potentials used. The current density values in the LiCl substituted group are statistically lower than control values, at all potentials at and above 0 mV ($P < 0.05$; indicated by an asterisk). C, The normalized conductance–voltage curve for the Na^+-sensitive outward current was plotted using the Boltzmann fit. The $V_{1/2}$ and slope value of the curve were -2.3 ± 1.4 mV and 8.7 ± 1.1, respectively.

4.4.5 Inhibiting Li^+ currents has no effect on outward currents

If Li^+ influx fails to activate K_{Na} currents, as has been reported (Hess *et al.*, 2007; Cervantes *et al.*, 2013), inhibition of Li^+ influx would be expected to have no effect on evoked K^+ currents. I tested this by bath application of TTX on to MNCs bathed in LiCl and found that TTX inhibited the inward currents but had no significant effect on the outward currents in any of the six MNCs tested (Figure 4.4). These data are consistent with the hypothesis that Li^+ substitution prevents activation of the same Na^+-activated K^+ current that is prevented by blocking Na^+ influx with TTX.

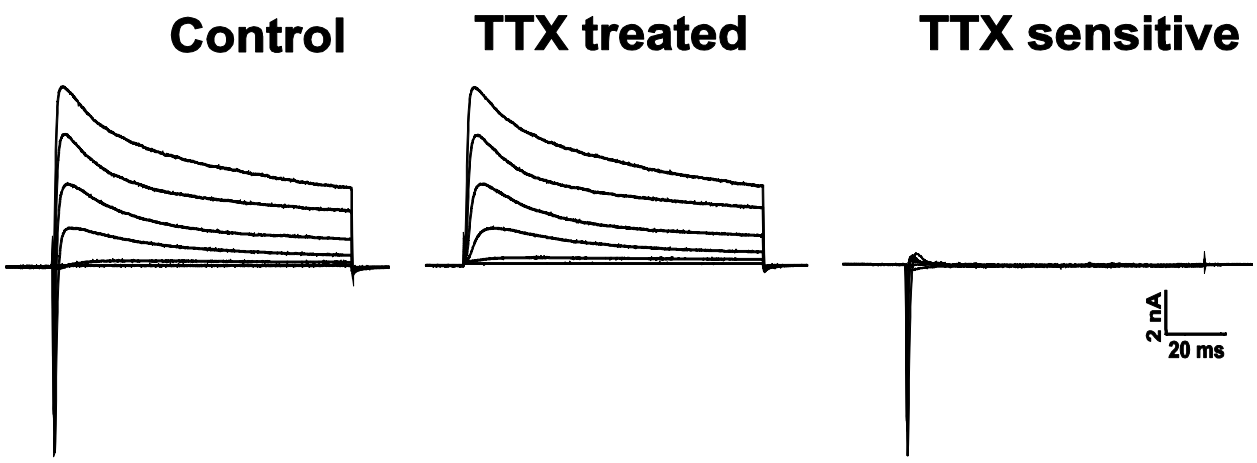

Figure 4.4. TTX has no effect on outward currents in acutely isolated rat MNCs bathed in LiCl solution. Whole-cell recordings showing representative currents evoked by stepping from −40 to +40 mV in 20 mV increments every five seconds from a holding potential of -70 mV. The internal and external solutions used in these experiments are described in the Materials and Methods section. 'Control' refers to currents evoked and recorded in an MNC bathed in an external solution in which NaCl has been iso-osmotically replaced by LiCl and 'TTX treated' refers to currents evoked and recorded in the same MNC after the 1 µM TTX bath application. The digitally subtracted currents show the 'TTX sensitive' currents for the two conditions.

4.4.6 Intracellular Na^+ loading increases evoked outward currents

The prevention of Na^+ influx by either the addition of TTX or the substitution of NaCl in the external solution with LiCl suggests that MNCs express a Na^+-activated K^+ current. I therefore hypothesized that increasing intracellular Na^+ should cause an increase in evoked outward currents in rat MNCs. I recorded outward currents in two separate sets of acutely isolated rat MNCs with patch pipettes that contained either 0 mM or 60 mM Na^+, as was done to investigate K_{Na} currents in olfactory and auditory neurons (Yang *et al.*, 2007; Lu *et al.*, 2010). I included 1 µM TTX in my external solutions to block Na^+ channels, which ensures that the activation of K_{Na} currents depended on the levels of Na^+ included in the pipette solutions rather than on the Na^+ flowing into the cells during the depolarizations (28). I also included 1 mM TEA in my external recording solutions to block as much non-K_{Na} K^+ current as possible (both Slack and Slick are reportedly insensitive to 1 mM TEA; (Bhattacharjee *et al.*, 2003). The internal pipette solutions were also modified. The 0 mM intracellular Na^+ solution contained (in mM) 70 KCl, 60 LiCl, 5 EGTA, and 10 Hepes (pH 7.2). The 60 mM intracellular Na^+ solution was the same except that the 60 mM LiCl was replaced with 60 mM NaCl. Note that the amplitudes of the control outward currents are smaller in this experiment than in the other experiments because of the presence of TEA in the external solution and because of the decreased gradient for K^+. In MNCs recorded with 60 mM Na^+ pipettes (n=12), I found that evoked outward currents were significantly higher than currents evoked in MNCs recorded with 0 mM Na^+ patch pipettes (n=12), suggesting the presence of an increased K^+ current component under Na^+ loaded conditions (Figure 4.5A). When the mean outward currents were normalized to cell capacitance, the outward current density in the cells with 60 mM intracellular Na^+ was found to be significantly higher than that in the cells with 0 mM intracellular Na^+ at potentials of 0 mV and above (Figure 4.5B). The mean outward current density

recorded in the 60 mM Na^+ and 0 mM Na^+ cells at -20 mV was 11 ± 2 pA/pF and 13 ± 4 pA/pF ($p > 0.05$), at 0 mV was 38 ± 2 pA/pF and 64 ± 9 pA/pF ($p < 0.05$), at 20 mV was 69 ± 4 pA/pF and 120 ± 14 pA/pF ($p < 0.05$) and at 40 mV was 91 ± 6 pA/pF and 155 ± 18 pA/pF ($p < 0.05$). I also plotted the normalized conductance-voltage curves for the increased outward currents observed in my Na^+ loaded conditions using values obtained by subtracting the basal conductance present at each tested potential in the two experimental groups. The $V_{1/2}$ of the increased outward currents so obtained was -6.8 ± 1.2 mV with 8.6 ± 1.0 as the slope of the Boltzmann fit (Figure 4.5C).

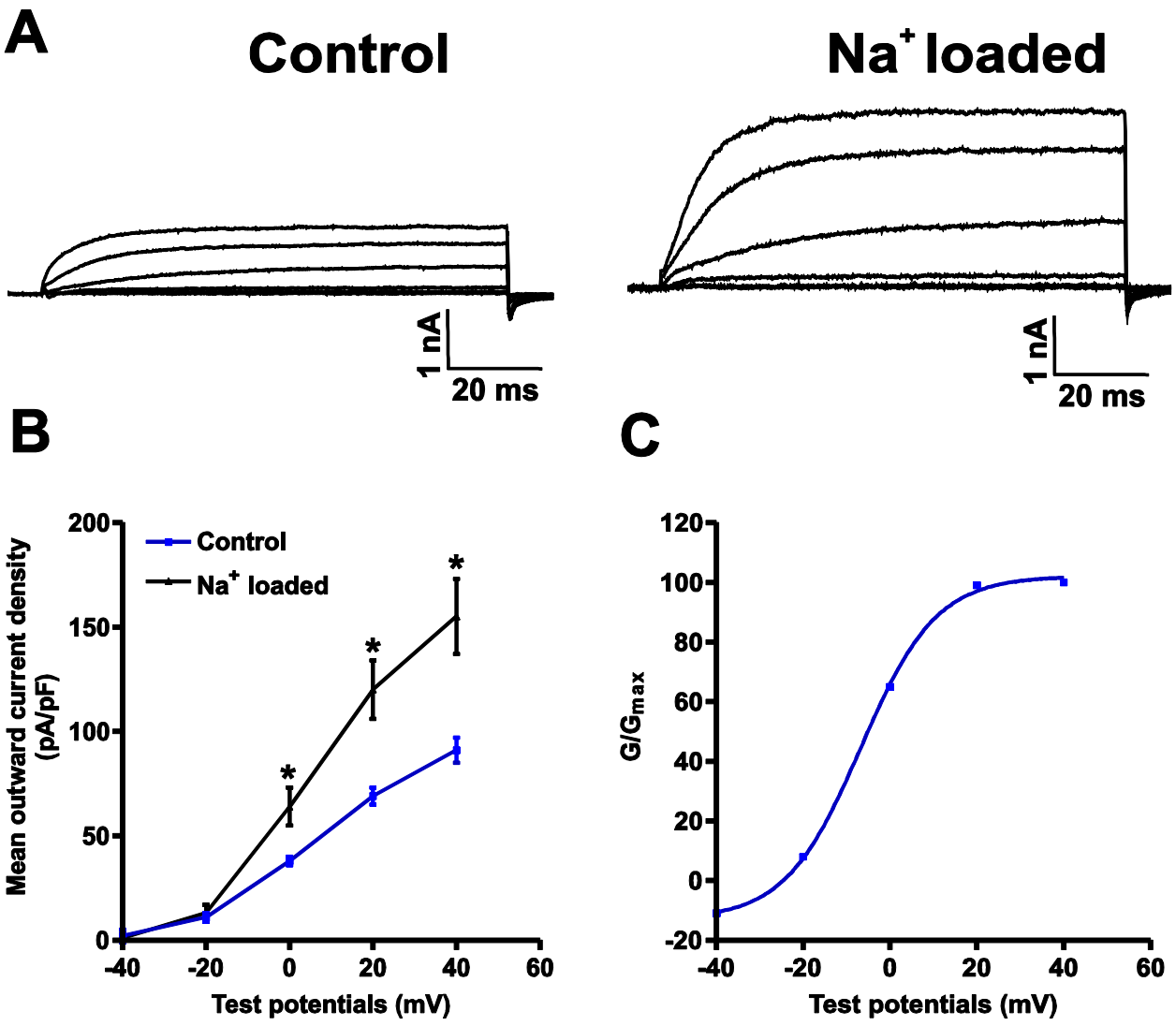

Figure 4.5. Intracellular Na^+ loading increases outward currents in acutely isolated MNCs.
A, Whole-cell recordings showing representative outward currents evoked by stepping from −40 to +40 mV in 20 mV increments every five seconds from a holding potential of -70 mV. The internal and external solutions used in these experiments are described in the Materials and Methods section. 'Control' refers to currents evoked in an MNC with 0 mM Na^+ in the patch pipettes and 'Na^+ loaded' refers to currents evoked in an MNC with 60 mM Na^+ in the patch pipettes. B, Mean current density of outward currents recorded in different MNCs under control and Na^+ loaded conditions are shown as a function of test potentials used. The current density values in the Na^+ loaded group are statistically higher from control values, at all potentials at and above 0 mV ($P < 0.05$; indicated by an asterisk). C, The normalized conductance–voltage curve for the increased outward current was plotted using the Boltzmann fit. The $V_{1/2}$ and slope value of the curve were -6.8 ± 1.2 mV and 8.6 ± 1.0, respectively.

4.5 DISCUSSION

The release of VP and OT is determined by the rate and pattern of firing of APs generated in the somata of the MNCs (Bicknell, 1988). The MNCs under isotonic or hypotonic conditions tend to be silent or fire infrequently in either an irregular or slow continuous manner. As plasma osmolality increases, however, the firing rate increases. This increase in osmolality may occur due to salt ingestion, insufficient water intake, or kidney malfunction. VP MNCs also tend to adopt a phasic firing pattern that comprises alternating periods of electrical activity (bursts) and silence each lasting for tens of seconds. OT MNCs on the other hand simply increase their firing rate during hyperosmotic conditions, although they do exhibit high frequency bursts during parturition and lactation (Brown *et al.*, 2013). Although the bursts observed in OT MNCs are usually shorter and of higher frequency than those seen in VP MNCs, burst firing in both types of MNCs maximizes the release of their respective neuropeptides. The mechanisms underlying burst firing in MNCs are incompletely understood, but it is thought that a variety of activity-dependent currents are involved (Brown *et al.*, 2013). MNCs display depolarizing afterpotentials (DAPs) whose summation is thought to contribute to the initiation of bursts and AHPs (afterhyperpolarizations) that are thought to contribute to the termination of bursts (Brown *et al.*, 2013). Rat MNCs exhibit 3 types of AHPs: an apamin-insensitive large amplitude fAHP that lasts less than 10 ms, an apamin-sensitive small amplitude AHP that lasts several hundred ms called the mAHP, and an apamin-insensitive very small-amplitude sAHP that lasts several seconds. The concerted interplay of these post-spike potentials with distinct amplitudes, time-courses, and mechanisms are thought to contribute to the generation of bursts of different frequencies and durations in MNCs (Brown *et al.*, 2013). AHPs in MNCs are largely mediated by activation of

different K_{Ca} channels, but the presence of K_{Na} channels and their contribution to the AHPs in MNCs has not been previously studied.

My immunocytochemical data suggest that rat MNCs express both Slick and Slack. Furthermore, my observation that both proteins are expressed in almost all OT and VP MNCs tested suggests that they are co-expressed in both types of MNCs. This raises the possibility that K_{Na} channels in rat MNCs might be formed of heteromultimers containing both Slick and Slack. Slick has been shown to form channels with Slack-B (but not Slack A) in an oocyte expression system (Chen *et al.*, 2009), but the possibility of heteromultimer formation between Slick and other Slack isoforms has not been tested. It remains to be determined which Slack isoforms are present in rat MNCs and if Slack and Slick form homomeric and/or heteromeric channels in rat MNCs.

I presented electrophysiological evidence demonstrating the presence of K_{Na} currents in rat MNCs. I have demonstrated that K_{Na} currents in rat MNCs are activated by Na^+ influx through voltage-dependent Na^+ channels and that adding TTX to block Na^+ influx through voltage-gated Na^+ channels or substituting external Na^+ with Li^+ caused significant reduction of outward currents in approximately 60-70% of the voltage clamped MNCs. I do not know why not all MNCs displayed a K_{Na} current, despite my observation that almost all MNCs express Slick and Slack proteins. The properties of the K_{Na} currents observed in rat MNCs are consistent with those reported in other cell types (Lu *et al.*, 2010; Cervantes *et al.*, 2013). I also demonstrated the presence of K_{Na} in rat MNCs by recording K^+ currents in MNCs using patch pipettes having either high (60 mM) or low (0 mM) levels of intracellular Na^+, as has been done in auditory and olfactory neurons (Yang *et al.*, 2007; Lu *et al.*, 2010). The evoked K^+ currents using step pulses under these two contrasting Na^+ conditions were directly compared and I found that the outward current

density recorded in cells with 60 mM Na^+ pipette was significantly higher at potentials equal to or greater than 0 mV. Inwardly rectifying K^+ channels have also been shown to be activated by high Na^+ levels (Ho *et al.*, 1999) and are expressed in MNCs (Li *et al.*, 2001; Del Burgo *et al.*, 2008). This type of current is unlikely to explain my results, however, because inwardly rectifying K^+ currents are not voltage dependent.

My data demonstrating the presence of K_{Na} channels in MNCs raises the possibility that they could contribute to the generation of AHPs in MNCs as they do in other neurons (Sanchez-Vives *et al.*, 2000; Liu *et al.*, 2004; Wallén *et al.*, 2007; Gao *et al.*, 2008). In lamprey spinal neurons, for example, sharp microelectrode recordings were used to show that one or more evoked APs activates an sAHP that is mediated by both K_{Ca} and K_{Na} currents (Wallén *et al.*, 2007). The size of both components is larger when the neurons are stimulated to fire a burst of APs and increases with the length of the burst (up to about 10 APs) and with the frequency of stimulation, presumably because of a greater accumulation of both Ca^{2+} and Na^+ inside the cell. Although this group originally reported that K_{Ca} currents accounted for about 80% of the total sAHP (Cangiano *et al.*, 2002), their later work suggested that the contribution of K_{Na} to the sAHP could be as large as 50% following high frequency bursts (Wallén *et al.*, 2007), which suggests that K_{Na} channels play an important role in shaping the firing behaviour of spinal neurons. My data demonstrates that MNCs express K_{Na} currents and suggests that they could contribute to the sAHP that gets activated during repetitive firing in MNCs. One study demonstrated that the sAHP following repetitive spiking in MNCs is largely (about 80%) mediated by K_{Ca} currents (Ghamari-Langroudi *et al.*, 2004). It is tempting to speculate increases in intracellular Na^+ could occur in the MNCs during repetitive spiking and that a resultant activation of K_{Na} current could contribute to the component of the MNC sAHP that is not dependent on K_{Ca}. Confirmation of a role for K_{Na} in the generation of

sAHPs in MNCs awaits further study.

Although the Na^+ concentration required for 50% activation (EC_{50}) of Slack and Slick in excised patch studies was found to be 41 mM and 89 mM respectively (Bhattacharjee *et al.*, 2003), it was later proposed that the binding of intracellular molecules like nicotinamide adenine dinucleotide (NAD) to Slack and Slick proteins might actually lower the Na^+ activation threshold of native K_{Na} channels and thus allow them to activate and participate under more physiologically relevant intracellular Na^+ changes (Tamsett *et al.*, 2009). Apart from the Na^+-dependent activation, the mechanism of which has recently been proposed for Slick channels (Thomson *et al.*, 2015), both Slack and Slick are also amenable to activation by a variety of neuromodulators through G protein-coupled receptor pathways (Santi *et al.*, 2006), which raises the possibility that K_{Na} channels in MNCs might also be directly regulated through synaptic inputs from other brain regions. Slack and Slick mediated currents have differences in their gating kinetics, their affinity for intracellular components like Cl^- and ATP, and their responses to neuromodulators (Bhattacharjee *et al.*, 2003; Bhattacharjee *et al.*, 2005a; Santi *et al.*, 2006). It remains to be determined whether and how neuromodulatory pathways might affect K_{Na} channels in MNCs.

My study provides the first evidence that functional Na^+-activated K^+ channels are present in rat MNCs. K_{Na} currents could contribute to AHP formation in the MNCs and thus play a role in determining MNC firing patterns and the release of VP and OT. It will be important to elucidate the function of these currents in MNC physiology and in systemic osmoregulation.

CHAPTER 5

GENERAL DISCUSSION

5.1 Osmotically-evoked PLC activation and PIP_2 decrease in acutely isolated rat MNCs.

As mentioned in the hypothesis and rationale section, the identity and role of second messenger systems in MNCs that could be activated in an osmosensitive fashion are not very clear. In the first part of my Ph.D. project, I was therefore interested in testing if MNCs exhibit osmosensitive changes in the intracellular PLC signalling pathway and if this contributes to the osmotic physiology of MNCs.

PLC signalling pathway has often been studied using transgenically expressed-PIP_2 binding fluorescent probes, in cultured cells (Stauffer *et al.*, 1998; Horowitz *et al.*, 2005; Lukacs *et al.*, 2013; Borbiro *et al.*, 2015). These probes are designed with a characteristic PLC δ1 PH (pleckstrin homology) domain that has high affinity for PIP_2 (Garcia *et al.*, 1995) and IP_3 (Horowitz *et al.*, 2005) molecules. This allows the PIP_2 binding probes to track PLC activation events in real time by monitoring fluorescence changes in stimulated cells. Since the membrane PIP_2 levels are relatively higher in unstimulated cells, the probes are initially seen localized in the cell periphery. However, upon stimulation of PLC pathway (for example by GPCR agonist treatment), these probes rapidly move from the membrane into the cell cytoplasm due to intracellular diffusion of the newly generated IP_3 molecules. The PLC activity can be quantified by measuring the time profile and reduction in the membrane PIP_2 fluorescence before and after the PLC activation by GPCR agonist. Although these probes are great tools to visualize the real time PIP_2 dynamics in live cells, their use for quantifying PIP_2 changes is limited because these PIP_2 probes can themselves interfere in the PLC action by sequestering the PIP_2 molecules and thereby reducing the PIP_2 availability for PLC action (Raucher *et al.*, 2000; Kwik *et al.*, 2003; Chun *et al.*, 2010). Moreover not

all cells, especially adult mammalian neurons like MNCs, are good candidates for transgenic expression of these probes (Karra *et al.*, 2010). PIP_2 changes in cells have also been measured using HPLC (high performance liquid chromatography) methods (Li *et al.*, 2005; Yamamoto *et al.*, 2006), in which cells are subjected to different experimental conditions and then the PIP_2 is extracted from these cells using a multi-step organic solvent treatment protocol. Although this method has the advantage that it could allow simultaneous profiling of many other phospholipids along with PIP_2, it is more appropriate for cultured cells that are plated at a very high density, unlike isolated rat MNCs, which typically yield only 500-1000 MNCs per animal (Oliet *et al.*, 1992).

To circumvent these issues and to be able to study PIP_2 changes in response to different treatments in acutely isolated MNCs, I therefore employed a relatively simple immunocytochemical approach using a PIP_2-specific antibody that has been previously used to demonstrate membrane PIP_2 changes (Hammond *et al.*, 2006). Since the PIP_2 antibody is introduced into the fixed cells after the cells have undergone PLC signalling events, this method has the advantage that it does not interfere with the PIP_2 dynamics by causing the PIP_2 shielding as is the case with PIP_2 probes in live cell imaging and therefore this method has the potential to allow better estimation of cellular PIP_2 changes in response to different treatments (Hammond *et al.*, 2006). Using this technique I was able to demonstrate that acutely isolated rat MNCs that are exposed to hypertonic saline for a short period (2-5 minutes) have a significantly smaller mean membrane PIP_2 immunostaining in comparison to cells that were maintained in isotonic saline. This osmotically-evoked decrease in membrane PIP_2 was prevented by pre-treatment of MNCs with U73192 (a PLC inhibitor), which confirms that PLC activation mediates the osmotically-evoked PIP_2 decrease in MNCs (Shah *et al.*, 2014). To my knowledge this was the first study to show osmotically-evoked PLC signalling in MNCs.

Studies have shown that isolated MNCs can rapidly shrink (within 2-3 minutes) and then remain shrunk for up to 10 minutes during acute hypertonic challenges due to absence of acute volume regulatory mechanisms (Zhang *et al.*, 2003b; Zhang *et al.*, 2008). This osmotically-evoked volumetric shrinkage allows the SICs, present in the MNC membrane, to rapidly activate and thus facilitate excitatory electrical changes in the MNCs, which promotes the systemic release of VP and OT. Furthermore, it has been shown that MNCs can transduce these osmotic challenges in a dose-dependent and reversible fashion (Oliet *et al.*, 1993b; Oliet *et al.*, 1994), which allows the MNCs to adjust their activity and hormonal release in accordance with the prevailing osmotic conditions in both graded and dynamic fashion. Using the PIP_2 immunocytochemical model, I showed that the osmotically-evoked PIP_2 decrease in rat MNCs occurs rapidly (in as early as 2 minutes), is reversible, and also occurs in a dose-dependent manner. The rapid, dose-dependent and reversible nature of this osmotically-evoked PLC effect are therefore consistent with the intrinsic osmosensitive properties of the isolated MNCs.

Previous studies have shown that osmotic threshold of isolated MNCs is quite low and they could detect and respond to osmotic changes of as little as ±3 mosmol kg^{-1} (Oliet *et al.*, 1994). Although the rapid and reversible nature of the osmotically-evoked PLC effect suggest that it could be an important component of MNC response during physiological osmotic perturbations, the lack of a significant PIP_2 decrease effect at smaller hypertonic dose (305 mosmol kg^{-1}) suggests otherwise and leads to two different possibilities. First, it is possible that osmotic activation of PLC observed by me might not be physiologically important during subtle osmotic changes that occur in the body on a daily basis such as during moderate exercising, and dehydration (Nose *et al.*, 1988; Edwards *et al.*, 2007) and eating salt (Andersen *et al.*, 2000), which typically causes only small (+5 to +10 mosmol kg^{-1}) increases in plasma osmolality. If this is true then osmotic activation

of PLC might be a specialized osmotic event that is activated only when MNCs experience higher than usual osmotic changes under chronic physiological conditions such as diabetes (Pasquel *et al.*, 2014) and kidney disease (Muhsin *et al.*, 2016), which causes larger (+20 mosmol kg^{-1}) increases in plasma osmolality. Second, the lack of PIP_2 effect at smaller hypertonic dose could be due to limitation of my PIP_2 quantification technique, which is based on averaged membrane fluorescence measurements in a population of cells that have undergone a similar treatment. It is therefore possible that this technique was not sensitive enough to detect subtle PIP_2 changes that might have occurred at smaller hypertonic doses, which suggests that osmotic activation of PLC could be a physiological important event in the MNC physiology even during daily osmotic changes. The true sensitivity and time course of the osmotically-evoked PLC signalling in MNCs is presently unclear. Further experiments such as live imaging in MNCs expressing better fluorescent probes that won't interfere in the PIP_2 signalling, whenever available, could possibly help resolve these issues.

I have also shown that osmotically-evoked PIP_2 decrease was prevented when MNCs were challenged with hypertonic saline in the presence of SB-366791 (a TRPV1 antagonist) and/or with hypertonic saline from which extracellular Ca^{2+} was removed, which suggests that osmotic activation of PLC occurs downstream of osmotic activation of TRPV1 channels and that it requires Ca^{2+} influx for activation. Since osmotic activation of TRPV1 channels in MNCs causes depolarization of MNCs (Sudbury *et al.*, 2010), I further tested if Ca^{2+} influx through depolarization-mediated opening of VDCCs could also contribute to the PIP_2 decrease observed by me.

VDCCs are broadly divided into high threshold (HVA) and low threshold (LVA) Ca^{2+} channel families, members of which can be distinguished based on their biophysical properties. The HVA

Ca^{2+} channel family encompasses L-, N-, P/Q- and R-type Ca^{2+} channels that mostly activate at high membrane depolarizations (Joux *et al.*, 2001), which are typically reached only during APs in neurons. Although LVA Ca^{2+} channels are typically represented by the T-type Ca^{2+} channels (Perez-Reyes, 2003), which activate at low membrane depolarizations that are below the AP threshold, it has been shown that L-type Ca^{2+} channels could also contribute to LVA Ca^{2+}currents in many cell types (Lipscombe *et al.*, 2004). The role of L-type Ca^{2+} channels in mediating LVA Ca^{2+} currents in isolated MNCs was first demonstrated by using nifedipine which is a dihydropyridine blocker (Fisher *et al.*, 1995) and is considered to be specific pharmacological blocker for L-type Ca^{2+} channels. Although the molecular identity of L-type LVA Ca^{2+} channels in MNCs is not clear, RT-PCR and immunostaining techniques have presented evidence for the presence of $Ca_V1.3$ (α1D) protein in MNCs (Glasgow *et al.*, 1999; Joux *et al.*, 2001), which is thought to form the LVA L-type Ca^{2+} channels in other cell types (Lipscombe *et al.*, 2004).

Since MNCs have been shown to express both HVA and LVA Ca^{2+} currents (Fisher *et al.*, 1996), I first tested if Ca^{2+} influx through HVA channels contributes to the PLC-mediated PIP_2 decreases. For this, I perfused a subset of MNCs with hypertonic saline solutions containing TTX to inhibit AP generation and thus participation of all activity-dependent HVA Ca^{2+} channels, but found no significant difference in the PIP_2 response in comparison to MNCs that were exposed only to hypertonic saline. This suggests that HVA Ca^{2+} channels might not be necessary for the osmotically-evoked PLC effects in MNCs under these experimental conditions. I then perfused a subset of MNCs with hypertonic saline that contained nifedipine. Since the osmotically-evoked PIP_2 decrease was prevented in the nifedipine-containing hypertonic group, it suggests that L type Ca^{2+} activation is necessary for the osmotically-evoked PIP_2 response. Together the TTX and nifedipine experiments suggest that Ca^{2+} influx through dihydropyridine-sensitive LVA Ca^{2+}

channels is sufficient to activate the Ca^{2+}-dependent PLC pathway in MNCs under these experimental conditions. The fact that the osmotically evoked PLC activation was maximally inhibited at relatively high (30μM) nifedipine concentrations, lend support to the idea that dihydropyridine-sensitive LVA Ca^{2+} currents could be mediated by $Ca_V1.3$ (α1D) channels, as they are relatively less sensitive to dihydropyridine blockers in comparison to other L type Ca^{2+} channels (Lipscombe *et al.*, 2004).

Since PLC signalling pathways regulate the functioning of many ion channels (Hilgemann *et al.*, 2001; Suh *et al.*, 2008a) including TRPV1 channels (Rohacs, 2007), I tested if the osmotically-evoked PLC pathway could affect the TRPV1 channels in the MNCs. The role of PIP_2 signalling in regulating TRPV1 channel behaviour has been controversial (Rohacs, 2007) and seems to vary with experimental conditions and cellular systems. Using whole cell patch methods, I found that osmotically- evoked TRPV1 currents were significantly smaller when the MNCs were pre-treated with a PLC inhibitor and/or a PKC inhibitor. The osmotically-evoked currents were however not affected when MNCs were loaded with exogenous PIP_2 through recording patch pipettes, which suggested that effect of PLC activation on TRPV1 opening is not directly mediated by decreases in membrane PIP_2 levels but rather is mediated by PKC activation. The overall summary of these electrophysiology experiments is that MNCs possess an osmotically-evoked PLC/PKC mechanism that could be rapidly activated and then acts in a feed forward manner to potentiate the osmosensitive TRPV1 currents. To my understanding, this is the first evidence of contribution of an osmotically-evoked enzymatic pathway in regulating the ion channel behaviour and the intrinsic osmosensitivity of MNCs.

Since TRPV1 channels are commonly known to desensitize after both an acute and long term agonist-evoked stimulation (Touska *et al.*, 2011), it is unclear how osmotically-evoked TRPV1

currents in MNCs could avert their desensitization and continue to stay active even during prolonged hypertonic conditions. Although there are reports suggesting that TRPV1 desensitization could occur in a Ca^{2+} independent manner (Tominaga *et al.*, 1998; Rosenbaum *et al.*, 2004), it is mostly the Ca^{2+} influx through TRPV1 that has been shown to cause TRPV1 desensitization through different pathways which includes direct Ca^{2+} dependent binding of calmodulin (a Ca^{2+} binding protein) to the TRPV1 channels (Numazaki *et al.*, 2003), Ca^{2+}-calmodulin dependent activation of calcineurin (a phosphatase) that could cause dephosphorylation TRPV1 channels (Mohapatra *et al.*, 2005) and Ca^{2+} dependent TRPV1 endocytosis that reduces surface expression of TRPV1 channels (Sanz-Salvador *et al.*, 2012). The absence of desensitization in osmotically-evoked currents in MNC TRPV1 variant therefore seems to be physiologically necessary to keep them active and maintain higher VP and OT levels during osmotic activation. One of the mechanism that has been proposed to facilitate sustained TRPV1 activation in MNCs is the absence of a characteristic N terminal domain in the MNC TRPV1 channels (Zaelzer *et al.*, 2015), which is responsible for wild type TRPV1 desensitization as it contains the calmodulin binding site (Lishko *et al.*, 2007). However as there have been reports suggesting that C terminal domain could also contribute to TRPV1 desensitization (Numazaki *et al.*, 2003; Lau *et al.*, 2012; Joseph *et al.*, 2013), which is present in the MNC TRPV1 channels (Zaelzer *et al.*, 2015), it is unclear if absence of N terminal domain is sufficient to confer the absence of TRPV1 desensitization mechanisms in MNCs. It is therefore likely that MNCs exhibit additional mechanisms that facilitates sustained osmotic activation of the TRPV1 channels. My PKC mediated potentiation of TRPV1 currents are therefore consistent with the presence of these additional mechanisms. A similar PKC mediated sensitization of TRPV1 currents in nociceptive neurons (Mandadi *et al.*, 2004; Mandadi *et al.*, 2006) has been previously shown, which lends

support to my observations. Although the true mechanism of PLC-PKC mediated potentiation of TRPV1 channels in MNCs is presently unknown, the N terminal variant of TRPV1 that confers intrinsic osmosensitive properties to MNCs has been shown to contain phosphorylation sites that could be targeted by PKC (Zaelzer *et al.*, 2015). It is therefore likely that osmotic activation of PLC-PKC phosphorylates the TRPV1 channels at one or more of those sites, which could sensitize them and keep them active during longer osmotically stimulated conditions. Alternatively, it is possible that osmotically-evoked PLC-PKC effect on TRPV1 channels in MNCs could be mediated by cytoskeleton proteins such as actin whose polymerization by angiotensin mediated GPCR-activation has been shown to facilitate TRPV1 currents (Zhang *et al.*, 2008). Whether or not PKC causes TRPV1 opening by phosphorylation or cytoskeleton rearrangement, the fact that it act to potentiate the intrinsic osmosensitivity suggest that osmotic activation of PLC is an important event in the MNC physiology and its electrical behaviour.

Besides regulation of TRPV1 channels, we have also utilized the osmotically-evoked PLC findings to demonstrate that osmotic activation of PLC plays an important role in hypertrophy of isolated MNCs that typically develops in tens of minutes during continuous exposure of MNCs to hypertonic conditions (Shah *et al.*, 2014). Although hypertrophy is an *in situ* structural and functional adaptation of MNCs that develops during prolonged hypertonic conditions in the body (Miyata *et al.*, 2002), the fact that some part of it could be replicated in isolated MNCs and that osmotic activation of PLC contributes to this rapid *in vitro* phenomenon suggests that the PLC pathway could also be a key player in regulating *in situ* hypertrophy. For example, previous studies have shown that MNC hypertrophy that develops after chronic hypertonic conditions is also characterized by increased surface expression of many ion channels and receptors like Na^+ channels (Tanaka *et al.*, 1999), Ca^{2+} channels (Zhang *et al.*, 2007a), NMDA receptors (Decavel *et*

al., 1997), dynorphin receptors (Shuster *et al.*, 1999) and VP receptors (Hurbin *et al.*, 2002), which are all probably needed for sustained excitability of the MNCs that could maintain enhanced VP and OT levels during chronic dehydrating conditions (Windle *et al.*, 1993). Since the cellular mechanisms regulating these functional changes in MNCs during hypertrophy are not well understood, there remains a possibility that osmotically-evoked PLC activation could contribute to some or all of these effects.

5.2 Na^+-activated K^+ channels in rat supraoptic neurons

As discussed in the hypothesis and rationale section, the presence of K_{Na} channels in MNCs was unknown. As the second part of my Ph.D. project, I was therefore interested to test if rat MNCs express functional K_{Na} channels and the commonly known K_{Na} proteins.

The molecular identity of native K_{Na} channels is not known but their biophysical properties are consistent with those of expressed Slick (slo 2.1) and Slack (slo 2.2) proteins and hence the presence of slack and slick proteins is considered to be an indication for the presence of K_{Na} channels (Bhattacharjee *et al.*, 2005a). Slack and Slick proteins are closely related to each other and also share sequence similarities with K_{Ca} channels (Bhattacharjee *et al.*, 2005a). Although Slick is only known to exist as a single isoform, recent studies have shown that Slack could exist in five different isoforms generated by alternate splicing of the Slack gene (Brown *et al.*, 2008). Although previous studies have shown that Slick is expressed (Bhattacharjee *et al.*, 2005b) while Slack B is absent in the SON (Bhattacharjee *et al.*, 2002), there remained a possibility that other Slack isoforms could be present in the SON and that MNCs could express functional K_{Na} channels.

Using immunocytochemical technique and primary antibodies that recognize all five known Slack isoforms (Brown *et al.*, 2008) and the only known Slick isoform, I found that both Slack and Slick proteins are expressed in the large majority of OT and VP rat MNCs. This raised the

possibility that functional K_{Na} channels could be present in the MNCs and that both Slack and Slick could contribute to forming these channels.

To test the functional presence of K_{Na} channels, I therefore employed electrophysiology whole-cell patch clamp techniques. Since pharmacological modulators for Slack and Slick channels are not specific, I used more common methods that relies on modifying the intracellular Na^+ levels, as have been done for demonstrating the presence of K_{Na} channels in other cell types (Yang *et al.*, 2007; Lu *et al.*, 2010; Cervantes *et al.*, 2013). Using these methods, I found that inhibition of Na^+ influx either by bath application of TTX (a Na^+ channel blocker) or iso-osmotic substitution of external Na^+ with Li^+ (Li^+ is known to permeate Na^+ channels without activating the K_{Na} channels) causes a significant reduction of outward K^+ currents in about 60% of the voltage- clamped MNCs. By varying the Na^+ amount in my recording patch pipettes, I have also shown that outward K^+ current density was significantly higher in rat MNCs, which were recorded with patch pipettes containing 60 mM Na^+ in comparison to rat MNCs, which were recorded with patch pipettes containing 0 mM Na^+. The presence of these Na^+-dependent outward K^+ currents in MNCs, is consistent with the functional evidence for K_{Na} channels in other cell types. To my understanding this is the first report that demonstrates that functional K_{Na} channels are expressed in rat MNCs.

Although the extent of the intracellular Na^+ increase that could occur in the MNCs during electrical activity is presently unknown, previous studies in other neurons have shown that both single AP spikes and repetitive electrical activity can cause high localized Na^+ levels near the cell membrane for tens of seconds (Bhattacharjee *et al.*, 2005a). Ratiometric imaging with Na^+-sensitive dyes like SBFI (sodium-binding benzofuran isophthalate) loaded into neurons, for example, has shown localized increases to nearly 5 mM after a single AP spike and up to 50-100 mM after multiple APs (Rose *et al.*, 2001; Zhong *et al.*, 2001). Although the Na^+ concentrations

required for 50% activation (EC_{50}) of Slack and Slick in excised patch studies was found to be 41 mM and 89 mM (Bhattacharjee *et al.*, 2003), respectively, the binding of intracellular molecules such as nicotinamide adenine dinucleotide (NAD) to Slack and Slick proteins have been shown to lower this activation threshold to about 20 mM, which suggests that native K_{Na} channels could activate and participate under much lower and physiologically relevant intracellular Na^+ changes (Tamsett *et al.*, 2009). Furthermore, the facts that there are localized high Na^+ signals during electrical activity in the neurons and K_{Na} channels could be activated by Na^+ influxes through voltage dependent sodium channels (VDSCs) even when recorded with patch pipettes containing 0 mM Na^+, suggests that K_{Na} channels could be activated by sensing Na^+ increases in their local environment. A possible physiological significance of this localized Na^+ sensing by K_{Na} channels could be their contribution to dampening of the neuronal over-excitability that could occur due to Na^+ permeable AMPA receptors during synaptic events which usually mediate only very small ionic currents. A similar close functional association of AMPA mediated Na^+ influx and K_{Na} channels have been shown to exist in the lamprey spinal cord neurons (Nanou *et al.*, 2007) suggesting that K_{Na} channels could also modulate subtle EPSCs in neurons that are needed for driving the threshold for neuronal activity. Although both Slack and Slick have been shown to have conserved Na^+ sensor motifs that are thought to allow direct binding of the Na^+ ions (Thomson *et al.*, 2015), there remains a possibility that other intracellular co-factors or binding partners like NAD^+ could be present and contribute to Na^+-dependent biophysical functioning of K_{Na} channels in MNCs and other cell types.

As mentioned in the introduction, MNCs exhibit three different activity-dependent AHPs (Armstrong *et al.*, 2010; Brown *et al.*, 2013) that are considered important for regulating their electric behaviour in physiological conditions. These outwardly rectifying AHPs in MNCs are

respectively characterized by their fast (fAHP), intermediate or medium (mAHP) and slow (sAHP) activation kinetics. Although AHPs in MNCs are largely thought to be mediated by K_{Ca} channels (Armstrong *et al.*, 2010) that are activated by increases in intracellular Ca^{2+}, the role of K_{Na} channels, that are similarly activated by intracellular Na^{+} increases following the neuronal electrical activity and participate in generation of AHPs in other cell types (Bhattacharjee *et al.*, 2005a), has not been investigated in MNCs.

My electrophysiology data demonstrating the presence of K_{Na} channels in MNCs suggest that they could contribute to the generation of AHPs in MNCs as they do in other neurons (Sanchez-Vives *et al.*, 2000; Liu *et al.*, 2004; Wallén *et al.*, 2007; Gao *et al.*, 2008). For example, a large part of both the fAHP (Bourque *et al.*, 1985) and the sAHP (Ghamari-Langroudi *et al.*, 2004) are due to Ca^{2+}-dependent K^{+} conductances, but Na^{+}-dependent K^{+} conductance could contribute to the remaining AHP portion. A similar dual contribution of K_{Ca} channels (major) and K_{Na} channels (minor) in lamprey spinal neurons has been shown during generation of the sAHP (Wallén *et al.*, 2007). Although it was originally reported that K_{Ca} currents accounted for approximately 80% of the total sAHP (Cangiano *et al.*, 2002), subsequent work suggested that the contribution of K_{Na} to the sAHP could be as large as 50% following high-frequency bursts (Wallén *et al.*, 2007), which suggests that K_{Na} channels could play an important role in shaping the firing behaviour of spinal neurons in an activity-dependent fashion. Similar experiments in hypothalamic explants have shown that sAHP following about 25 spikes in MNCs is largely (approximately 80%) mediated by K_{Ca} currents (Ghamari-Langroudi *et al.*, 2004). The remaining non-K_{Ca} component, contributing to sAHP, has not been characterized and it is therefore possible that it could be mediated by K_{Na} channels. Moreover as MNCs usually exhibit much more than 25 spikes in their high frequency burst patterns (Brown *et al.*, 2013), it is quite possible that the K_{Na}

contribution to the sAHP in MNCs was underestimated by the 25 impulses experimental protocol that was used in this study. Alternatively the K_{Na} channels could contribute to generation of the fAHP that is needed to keep the MNCs in a refractory state and prepare them for subsequent AP spike. My data showing that MNCs express functional K_{Na} channels is therefore an important step towards further understanding of their AHP mechanisms. To fully ascertain the role of K_{Na} channels in contribution to the MNC electric behaviour further experiments are needed.

5.3 Future directions

The work presented in this thesis is useful for better understanding of MNC physiology and suggests a number of interesting research directions that could be pursued. Some of these future opportunities are listed below:

5.3.1 Future directions for PLC project

1) One of the key findings of this thesis is the demonstration of a novel osmotically-evoked and Ca^{2+} dependent PLC pathway in rat MNCs that acts to potentiate their intrinsic osmosensitivity. Since PLC δ1 and PLC δ4 are the only two highly Ca^{2+} sensitive isoforms reported in SON (Hazell *et al.*, 2012), and my initial experiments have suggested that PLC δ1 might not be involved in the osmotically-evoked PLC effects, our laboratory is now interested in testing the potential role of the PLC δ4 isoform. We have already initiated this process and are now waiting to receive PLC δ4 knockout mice from our collaborator, Dr. K. Fukami. Once we have the PLC δ4 animals, our laboratory will test those animals by performing PIP_2 experiments to check if the osmotically-evoked PIP_2 decrease response in those MNCs is inhibited or is any different from the regular wild-type controls. If we find any significant difference in the osmotically-evoked PIP_2 response of these PLC δ4 knock out MNCs, we will test if PLC δ4 contributes to the osmotically-evoked hypertrophy response by comparing their hypertrophy response to that of wild type mice.

To test if the PLC δ4 isoform contributes to systemic osmoregulation in the body, we will also test these knockout animals for their ability to secrete VP and OT during chronic dehydrating conditions as have been done in the TRPV1 knockout mice (Sharif Naeini *et al.*, 2006). If PLC δ4 isoform is indeed important for hypertrophy and systemic osmoregulation, we would see a much lower incidence of hypertrophy and reduced plasma levels of VP and OT hormones in the knockout mice in comparison to the wild-type control mice. Finally, as the MNCs are primarily destined for on-demand exocytotic release of VP and OT hormones into the systemic circulation (Bourque *et al.*, 1997; Brown *et al.*, 2013), *the* involvement of PLC signalling components in principle could contribute to a variety of intracellular events such as transcriptional activation of hormone synthesis, protein translation, trafficking of hormones into the exocytosis zone, packaging of hormones into release vesicles, facilitating docking and recycling of vesicles for efficient release into the pituitary (Martin, 2012). It would therefore be further interesting to test the mechanistic contribution of the PLC isoforms in regulating these intracellular events.

2) Besides TRPV1 channels, MNCs have a large repertoire of other ion channels such as HCN (Hyperpolarization-activated cyclic nucleotide-gated channels) (Ghamari-Langroudi *et al.*, 2000), K_{ATP} channels (Song *et al.*, 2014), K_{ir} channels (Karschin *et al.*, 1996; Han *et al.*, 2003), TRPM channels (Teruyama *et al.*, 2011) and K_v7 channels (Zhang *et al.*, 2009), which have been shown to respond to PIP_2 changes in other cell types (Hilgemann *et al.*, 1996; Liu *et al.*, 2003; Xiao *et al.*, 2003; Nilius *et al.*, 2006; Brown *et al.*, 2007; Pian *et al.*, 2007). Based on my osmotically-evoked PLC activation observations, it is tempting to speculate that some or all of these channels could be regulated by the osmotically-evoked and/or receptor mediated PLC signalling mechanism in the MNCs. It will therefore be interesting to test the role of PIP_2 pathway in sustaining the electrical behaviour of these channels in MNCs using a combination of pharmacological and

electrophysiology techniques. This will potentially help in further understanding of MNC physiology and the regulation of MNC electrical behaviour by the PLC signalling pathway.

3) Although the work contained in thesis has demonstrated that there are osmotically-evoked and receptor-evoked changes in membrane PIP_2 levels, there remains a possibility that other membrane phospholipids like PI4P, which are also known to be hydrolyzed by PLC to generate second messengers (Serunian *et al.*, 1989; Delage *et al.*, 2013; Tan *et al.*, 2014), could also have contributed in the PLC mediated ion channel and hypertrophy effects. The contribution of other membrane lipids in the PLC mediated MNCs physiology remains to be tested and would be an interesting research opportunity.

4) MNCs are known to exhibit increase in their cell size during chronic dehydrating conditions through a process called hypertrophy, which is a structural and functional adaption that helps them secrete more VP and OT during chronic osmotic perturbations (Miyata *et al.*, 2002) . The exact mechanisms for this process are not well understood. Recently, we have shown that at least some part of this hypertrophy response is due to PLC-dependent exocytotic fusion of intracellular vesicles on to the plasma membrane of MNCs (Shah *et al.*, 2014b). Since insertion/addition of these new vesicle membranes into the existing cell membrane during hypertrophy might decrease the mechanical tension experienced by the existing SICs, this in principle should inhibit their activity. However as MNCs are active even during chronic dehydrating conditions as seen by high plasma VP and OT levels (Windle *et al.*, 1993; Greenwood *et al.*, 2015), it is tempting to speculate that the osmotic activation of PLC/PKC pathway could be contributing to the sustained SIC activity of hypertrophied MNCs by causing translocation of preformed SIC channels to the MNC membrane. A similar PKC-dependent rapid TRPV1 translocation mechanism has already been shown in expressed cell systems (Morenilla-Palao *et al.*, 2004). It would therefore be

interesting to test if SIC channels are indeed translocated to the MNC membrane during these conditions by using TRPV1 antibodies that could bind to extracellular epitopes of TRPV1 channels (Meng *et al.*, 2016). This can be done by incubating subsets of MNCs that have been fixed after a 90 min hypertonic treatment with this antibody and comparing the immunofluorescence of this group with that of control group MNCs that were treated with isotonic conditions during this period. Alternatively the SON blocks from dehydrated rats could be homogenized and TRPV1 staining in the membrane fractions obtained from the dehydrated rats could be compared to the ones obtained from control animals. If SICs channels are indeed translocated by osmotic activation of MNCs to facilitate the hypertrophic response, one would see significantly higher immunostaining for TRPV1 in membrane fractions prepared from dehydrated rats and/or isolated MNCs that were exposed to 90 min hypertonic stimulation.

5.3.2 Future directions for K_{Na} project

1) Slack is known to exist in five different isoforms (Brown *et al.*, 2008) including Slack B, which was earlier found to be absent in SON (Bhattacharjee *et al.*, 2002). Although my experiments using a non-isoform specific Slack antibody suggest that rat MNCs might express one or more of the non-Slack B isoforms (Bansal *et al.*, 2016), it is unclear which of them are actually present in the MNCs. It will therefore be important to identify the Slack isoforms that are present in MNCs using either RT-PCR techniques or isoform-specific antibodies.

Also my immunostaining experiments suggest that Slack and Slick proteins are present in most VP and OT MNCs, however, the true composition of native K_{Na} channels is still unclear (Bansal *et al.*, 2016). It is therefore possible that native K_{Na} channels could either be a) combination of Slack and Slick homomers or b) heteromers of Slack and Slick or c) a mix of different homomeric and heteromeric combinations of Slack and Slick proteins. To test if Slack

and Slick proteins are indeed heteromerizing in the MNCs, further experiments like co-immunoprecipitations of Slack and Slick proteins could be performed. This would be greatly aided by isoform-specific Slack antibodies if these are available in the future. For example, rat SON homogenates can be used in immunoprecipitation experiments with either Slack or Slick antibody and then probed for the presence of the other protein. If both proteins are structurally associated, the immunoprecipitated homogenate prepared with either antibody should show immunostaining for the other protein. This can also be tested by using Forster resonance energy transfer (FRET) based approach in native MNCs wherein Slack isoform specific antibodies can be tagged with a FRET donor fluorophore and Slick specific antibody can be tagged with a FRET acceptor fluorophore or vice versa. Thereafter the donor fluorophore can be excited and the emitted fluorescence from the acceptor fluorophore can be measured for possible functional proximity between the two proteins inside the MNCs.

2) Although my electrophysiology and immunostaining data in acutely isolated MNCs suggest that functional K_{Na} channels are expressed in rat MNCs and may contribute to the regulation of their electrical behaviour by facilitating AHP generations in an Na^+ influx-dependent fashion, it is important to test their role in regulating the MNCs excitability and firing patterns. This for example can be done by performing single unit extracellular or sharp microelectrode intracellular recordings in hypothalamic explants or slices, which are considered more appropriate models for studying the firing properties of MNCs. The role of K_{Na} channels can be tested for example by iso-osmotic substitution of NaCl with LiCl during current clamp experiments. Since Li^+ could permeate Na^+ channels to generate APs without activating K_{Na} channels, this experiment can potentially test if K_{Na} channels indeed contribute to the MNC firing events. To test if modulation of MNC firing pattern would have occurred through AHP modulation, fAHP generated after a single AP spike or

sAHP generated after a burst of MNC activity could be compared before and after LiCl substitution.

3) Although it is unknown if there are changes in the AHPs of MNCs during chronic hyperosmotic conditions, both the mAHP and the sAHP have been shown to increase in at least the OT MNCs of pregnant and lactating female rats as a possible protective adaptation to prevent their over-excitation (Teruyama *et al.*, 2005). It would therefore be interesting to test if changes in K_{Na} conductance also contributes to this hypothalamic plasticity that occurs under chronic physiologically-demanding conditions. For example, this can be done by performing electrophysiological recordings in MNCs of osmotically-challenged animals and comparing it with the control animals. If the K_{Na} conductance would indeed have increased, one would expect to see a much higher K_{Na} current component in the AHP of these experimental animals in comparison to recordings made in MNCs of control animals.

4) Although K_{Na} channels are more commonly associated with AHP mechanisms and thus contribute to activity-dependent changes in electrical behaviour of MNCs, previous study in lamprey spinal cord neurons have shown that K_{Na} channels could contribute to synaptic modulation of EPSCs (Nanou *et al.*, 2008). Since MNCs exhibit EPSCs in response to synaptic innervations from different brain regions such as OVLT (Richard *et al.*, 1995) and olfactory bulb (Yang *et al.*, 1995), it would be interesting to test if K_{Na} activation contributes to regulation of these inputs. Furthermore both Slack and Slick form voltage sensitive channels, which have been shown to respond to metabotrophic receptor activation through second messenger systems even in a Na^+ independent fashion (Santi *et al.*, 2006). This raises the possibility that native K_{Na} channels in MNCs could directly respond to synaptic neuromodulators like glutamate, angiotensin and acetylcholine that act on metabotrophic receptors present on MNCs. It would therefore be

interesting to decipher the physiological significance and contribution of K_{Na} channels in regulating the synaptic physiology of MNCs that could occur both in a Na^+-dependent and independent fashion.

5) Finally it is known that K_{Na} channels are not the only channels that are gated by changes in intracellular Na^+ levels. For example, ion channels like GIRK (G protein-coupled inwardly-rectifying potassium) (Ho *et al.*, 1999) and NMDA (Yu *et al.*, 1998) are also known to activate by increased intracellular Na^+ levels in other cell types. Since both GIRK channels (Li *et al.*, 2001; Del Burgo *et al.*, 2008) and NMDA (Pak *et al.*, 2002; Doherty *et al.*, 2011) channels are reportedly present in rat MNCs, it raises the possibility that they could also behave in a similar Na^+-sensitive fashion and thus contribute to MNC electrical behaviour. It would therefore be interesting to further test the Na^+-dependent regulation of these channels in MNCs by performing electrophysiology experiments.

5.4 Summary and Conclusion

The work presented in this Ph.D. thesis has demonstrated evidence for the presence of a novel osmotically-evoked PLC pathway in rat MNCs that is Ca^{2+}-dependent and acts to potentiate the osmosensitivity of MNCs in a feed forward manner. It has also been demonstrated in this thesis that rat MNCs express Na^+ activated K^+ (K_{Na}) channels that could potentially contribute to regulation of their electric behaviour. Overall the work contained in this thesis has identified mechanisms that could be important in determining the osmotic physiology of supraoptic neurons and the maintenance of body fluid homeostasis. This thesis work also has the potential to help us better understand the etiology of body fluid imbalance, which is one of the most common clinical problems in chronically ill and/or elderly patients.

5.5 Major thesis findings

1. Using a PIP_2 antibody based immunocytochemical model to quantify PIP_2 changes, acutely isolated rat MNCs were found to have a significantly weaker PIP_2 immunostaining after acute hypertonic saline exposure that suggested the presence of an osmotically-evoked PLC pathway in rat MNCs.

2. The osmotically-evoked PLC pathway in rat MNCs was found to be dependent on osmotic activation of SICs that are known to provide the depolarizing trigger to MNCs under hypertonic conditions.

3. The osmotically-evoked PLC pathway in rat MNCs was found to be dependent on Ca^{2+} influx that is mediated by dihydropyridine-sensitive L-type LVA Ca^{2+} channels.

4. Using electrophysiology methods, the osmotically-evoked PLC pathway was found to potentiate TRPV1-encoded SICs in a feed forward manner and thus contribute to the intrinsic osmosensitivity of rat MNCs.

5. Using electrophysiology methods, rat MNCs were also found to express Na^+- activated K^+ (K_{Na}) channels, which could contribute to the regulation of their electrical behaviour as in other neurons.

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