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The Role of the PGE_2/EP System in the Regulation of Na^+/K^+-ATPase Protein Levels and Cell Survival in the M-1 Cortical Collecting Duct Cell Line

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THE ROLE OF THE PGE_2/EP SYSTEM IN THE REGULATION OF Na^+/K^+-ATPase PROTEIN LEVELS AND CELL SURVIVAL IN THE M-1 CORTICAL COLLECTING DUCT CELL LINE

Geneviève Paris

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ABSTRACT

Prostaglandin (PG) E_2 is highly produced in the kidney and regulates sodium reabsorption, which is driven by the Na^+/K^+-ATPase (NKA) pump. It has been reported that high salt affects the activity of NKA in the cortical collecting duct. The current study addresses the role of PGE_2 in NKA modulation and we hypothesize that PGE_2 is acting via the EP_4 receptor to increase NKA protein levels in hypertonic conditions. We demonstrated by immunoblotting an increase in NKA steady state levels of protein and activity in hypertonic conditions. Hypertonicity also stimulates cyclooxygenase (COX)-2 and PGE_2 production and modulates EP receptor mRNA expression. Involvement of PGE_2 in NKA regulation was determined by COX inhibition (indomethacin and ibuprofen) and EP receptor antagonism (L-161 982 (EP_4), SC51089 (EP_1)). With the exception of L-161 982, no change in NKA protein levels was observed, suggesting that NKA regulation is independent of PGE_2 in high salt. Our results also indicated that PGE_2 is not involved in cell survival and that L-161 982 increases apoptosis, which can explain the decrease in NKA levels in high salt.

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LIST OF ABBREVIATIONS

AQP	Aquaporin
ATP	Adenosine triphosphate
AVP	Arginine-vasopressin
Bax	Bcl-2 associated X
Bcl-2	B-cell lymphoma 2
BSA	Bovine serum albumin
cAMP	Cyclic adenosine monophosphate
CCD	Cortical collecting duct
COX	Cyclooxygenase
DAG	Diacylglycerol
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dpm	Disintegration per minute
ENaC	Epithelial Na^+ channel
EP	E-prostanoid
FBS	Fetal bovine serum
GAPDH	Glyceraldehyde-3phosphate dehydrogenase
IBMX	Isobutylmethylxanthine
IP3	Inositol-1,4,5-trisphosphate
MAPK	Mitogen-activated protein kinase
MDCK	Madin-Darby canine kidney
mRNA	Messenger ribonucleic acid
MRP4	Multidrug resistance protein
NHE1	Na^+/H^+ exchanger
NKA	Na^+/K^+-ATPase pump
NSAID	Non steroidal anti-inflammatory drug
OMCD	Outer medullary collecting duct
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PGT	Prostaglandin transporter
PG	Prostaglandin
PGE_2	Prostaglandin E_2
PI3K	Phosphatidylinositol 3 kinase
PIP_2	Phosphatidylinositol-4,5-bisphosphate
ROMK	Renal outer medullary potassium channel
RTK	Receptor tyrosine kinase
TBS-T	Tris buffered saline-Tween 20

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1. INTRODUCTION

1.1 Kidney

The human body has developed remarkable and complex mechanisms to maintain its homeostasis. A very important player in this homeostatic regulation is the kidney. The principal role of the kidney is to maintain the composition and volume of extracellular compartments by secreting waste (urea, creatinine) and reabsorbing water and solutes. Another important role of the kidney is to produce specific hormones, such as erythropoietin (red blood cell production) and renin (blood pressure regulation). Furthermore, the kidneys possess a neoglucogenic function and take part in the metabolism of certain drugs (Anders 1980).

Every day, the kidneys filter around 180 liters of plasma and produce 1 to 2 liters of urine. The filtration occurs through the nephron, the structural and functional unit of the kidney. Each kidney is made of approximately one million nephrons. They are divided in different segments, named according to their location and functions. These segments consist of the glomerulus, the proximal tubule, the loop of Henle, the distal convoluted tubule, the cortical collecting duct and the outer and inner medullary collecting duct. Each section of the nephron is characterized by the presence of specific carriers that maintain the homeostasis of extracellular compartments, despite the large fluctuations in dietary intake of water and solute.

1.2 Cortical collecting duct

The cortical collecting duct (CCD), located in the final section of the nephron, is responsible for the fine-tuning of salt, water and acid/base transport in order to maintain a normal blood pressure, pH and electrolyte balance. The CCD epithelium is composed of two different cell types connected by tight junctions; the α/β-intercalated cells and the principal cells. The α/β-intercalated cells account for 40% of the CCD and are involved in H^+ and HCO_3^- transport. The α-intercalated cells are more abundant. These cells secrete H^+ via an H^+-ATPase transporter and reabsorb HCO_3^- via a Cl^-/HCO_3^- exchanger. Conversely, the β-intercalated cells secrete HCO_3^- and reabsorb H^+ (Emmons *et al.* 1991; Kim *et al.* 1999).

The principal cells **(Figure 2)** constitute 60% of the CCD and they are specialized in Na^+ reabsorption and K^+ secretion. This ion transport is driven by the Na^+/K^+-ATPase (NKA) pump, present at the basolateral membrane of principal cells. Using ATP, NKA actively transports three sodium (Na^+) ions outside the cell and two potassium (K^+) ions inside, maintaining a high K^+ and low Na^+ concentration inside the cell (Horisberger 2004). Following this gradient, Na^+ and K^+ will passively diffuse through the cell via the amiloride-sensitive epithelial sodium channel (ENaC) and the renal outer medullary potassium channel (ROMK) respectively (Stokes and Sigmund 1998). The Na^+ transport in CCD principal cells is regulated by many hormones and factors. For example, the release of aldosterone in the CCD increases ENaC mRNA expression and protein levels on the apical membrane of principal cells (Bens *et al.* 1999). Aldosterone also increases activity and expression of NKA in the CCD (Bens *et al.* 1999; Verrey *et al.* 1987; Verrey, Kraehenbuhl, Rossier 1989; Welling *et al.*

1993). The antidiuretic hormone arginine vasopressin (AVP) is another important regulator of salt transport in the CCD. AVP increases Na^+ transport in the CCD by up-regulating ENaC mRNA expression (Nicco *et al.* 2001) and by increasing cell surface expression and activity of NKA (Feraille *et al.* 2003). Other factors such as prostaglandins, insulin and dopamine also play important roles in the regulation of salt transport in the CCD, mainly by modulating NKA activity and levels. Their actions will be described in more detail in the next sections.

Principal cells are also involved in water reabsorption, which occurs via specialized water channels named aquaporins (AQP). The AQP2 channel is present at the apical membrane of CCD principal cells while the AQP3 and AQP4 channels are detectable at the basolateral membrane (Nielsen *et al.* 1999). These channels are stored in intracellular vesicles that are rapidly translocated to the cell membrane under specific conditions (Stetson, Wade, Giebisch 1980). For example, following a decrease in blood volume, AVP will be released from the pituitary gland and will rapidly induce the translocation of AQP2 to the cell membrane to reabsorb water (Nielsen *et al.* 1995). There is also emerging evidence that hypertonicity can induce the transcription of AQP2 via an AVP-independent pathway after 24 hours in high NaCl conditions (Hasler *et al.* 2006).

Moreover, the CCD principal cells express the Na^+/H^+ exchanger 1 (NHE1) which helps to maintain intracellular pH and volume (Hill, Giesberts, White 2002). The NHE1 isoform is present at the basolateral membrane and exchanges one H^+ for one Na^+ according to their transmembrane chemical gradients. Under normal conditions, Na^+ diffuses inside the cell and H^+ outside. NHE1 can be induced by

different stress stimuli, such as osmotic cell shrinkage (Pederson *et al.* 2002), mechanical stretch (Tominaga and Barber 1998) and hypoxia (Rios *et al.* 2005). It can also be regulated by hormones and growth factors (Wakabayashi, Shigekawa, Pouyssegur 1997). However, the mechanisms involved in NHE1 regulation in the CCD remain unclear.

1.3 Na^+/K^+-ATPase

The first publication describing the presence of an ATPase pump transporting Na^+ and K^+ across the cell membrane was presented in 1957 by a Danish scientist named Jens Christian Skou (Skou 1957). Forty years later, in 1997, he wins the Nobel Prize in Chemistry together with Paul D. Boyer and John E. Walker for his discovery of Na^+/K^+-ATPase (Lores Arnaiz 1998). The NKA pump is an integral membrane protein located exclusively at the basolateral side of epithelial cells (Schwartz *et al.* 1974). The main function of NKA is to transport Na^+ and K^+ across the cell. NKA is a heterodimer composed of subunits α and β. It can also be associated with a small third subunit named γ.

The α-subunit is the largest subunit of the complex (1000 amino acids, 112 kDa). This subunit possesses the catalytic activity; it is responsible for ATP hydrolysis and binding of Na^+ and K^+ ions. In mammals, there are four isoforms of the α-subunit (α_1-α_4) (Lingrel *et al.* 1990). The α_1-subunit is ubiquitously expressed and is found in high concentration in the kidney. The α_2 is present in the brain, heart, and skeletal muscles. The α_3 is expressed in neural tissue and ovaries while the α_4 is only present in the testis (Sweadner 1989; Woo, James, Lingrel 1999). The

α-subunit is composed of ten transmembrane domains, named M1 to M10, with intracellular N and C-terminal ends. The M4 and M5 transmembrane domains surround a long intracellular loop containing the ATP binding site and the catalytic phosphorylation site. The presence of an ion pore formed by the M4, M5 and M6 transmembrane domains is also suggested and the N-terminal end might be playing a role in gating this pore. The extracellular domain between the M7 and M8 transmembrane domains is involved in the interaction with the β-subunit. Finally, many additional phosphorylation sites have been identified along the α-subunit, mainly located at the N-terminal domain. These sites are thought to be involved in NKA regulation by different protein kinases. For example, NKA α-subunit can be phosphorylated at serine 943 by the protein kinase A (PKA) (Fisone *et al.* 1994), and at serine 11 and 18, by the protein kinase C (PKC) (Feschenko and Sweadner 1995). However, a lot of controversy exists regarding the effect of NKA phosphorylation by PKA. NKA activity can either be activated, inhibited or unaffected by PKA phosphorylation (Carranza *et al.* 1996; Cornelius and Logvinenko 1996; Fisone *et al.* 1994; Kiroytcheva *et al.* 1999). Conversely, phosphorylation by PKC seems to act as a signal for endocytosis and exocytosis of NKA (Carranza, Feraille, Favre 1996; Lopina 2001). However, further analysis is needed for a better understanding of NKA regulation by protein kinases.

Association of α and β-subunit is necessary for the formation of the active pump. Interaction with the β-subunit allows the proper folding of newly synthesized α-subunits, participates in the translocation of the α-subunit from the endoplasmic reticulum (ER) to the cell membrane and stabilizes the α-subunit within the cell

membrane (Geering 1991; Ueno *et al.* 1997). The β-subunit consists of a 370 amino acid sequence with a molecular weight of 33.6 kDa. However, it normally weights around 55 kDa due to the presence of three sites of extensive glycosylation (Farley, Miller, Kudrow 1986; Miller and Farley 1988). Three different β-isoforms have been identified in mammals (β_1-β_3). β_1 is ubiquitously expressed, β_2 is mainly found in skeletal muscles, pineal gland and neural tissue (Fowles, Green, Ouyang 2004; Lingrel and Kuntzweiler 1994; Malik *et al.* 1998) and β_3 is present in testes, retina, liver and lung (Arystarkhova and Sweadner 1997). In the kidney, the β_1-subunit is the most abundant (Lingrel and Kuntzweiler 1994). The β-subunit consists of a single transmembrane domain with an intracellular N-terminal end and a large extracellular C-terminal domain. This large extracellular domain is responsible for the interaction with the α-subunit (Jorgensen, Hakansson, Karlish 2003).

It is not clear whether the α or β-subunit is the rate-limiting step in the formation of the active pump. This regulation seems to be tissue specific. For example, studies have reported an excessive production of α-subunits in alveolar epithelial cells (Yoshimura *et al.* 2008) and skeletal muscles (Taormino and Fambrough 1990), making synthesis of the β-subunit the rate-limiting step. Conversely, the α-subunit seems more likely to be the limiting subunit in the collecting duct. In fact, studies performed on rat outer medullary collecting duct (OMCD) and Madin-Darby canine kidney (MDCK) cells have reported a higher level of β-subunit mRNA in the cells, suggesting that α-subunit is the rate-limiting step

(Buffin-Meyer *et al.* 1998; Tokhtaeva, Sachs, Vagin 2009). Further investigations are needed to evaluate the exact number of each subunit in CCD principal cells.

Finally, NKA can be associated with a γ-subunit (53 amino acids, 10 kDa). This subunit is not required for the functional activity of the pump but it can decrease its affinity for ATP, Na^+ and K^+ (Arystarkhova *et al.* 1999; Therien, Karlish, Blostein 1999). The γ-subunit is not expressed in the collecting duct under basal conditions. However, expression of γ-subunit has been detected in mouse inner medullary collecting duct (IMCD3) cells upon long-term adaptation to hypertonicity (Capasso, Rivard, Berl 2001). Further characterization is needed to determine if this up-regulation occurs in the CCD as well.

NKA is the largest member of the P-type ATPase family which transport ions across the cell membrane (Jorgensen, Hakansson, Karlish 2003). These proteins are also named E_1-E_2-ATPase, referring to the two possible conformational states of the pump. For NKA, the E_1 state has a high affinity for Na^+ and ATP and a low affinity for K^+, whereas E_2 has a high affinity for K^+ and a low affinity for Na^+. The pump is activated by transient phosphorylation of these two states by ATP. The sequence of activation of NKA is described with the Post-Albers model (E_1, E_1-P, E_2-P and E_2) (Albers 1967; Post *et al.* 1969). First, ATP and Na^+ that possess a high affinity for the E_1 state bind to the intracellular side of NKA. Binding of ATP phosphorylates the pump (E_1-P state) and occludes the Na^+ ions inside it. Next, the release of ADP allows the transition from the E_1-P state, to E_2-P. Na^+ is then released in the extracellular space and K^+ binds to NKA. The binding of K^+ leads to the dephosphorylation of the pump to become E_2. Finally, the K^+ ions are released

in the intracellular space following a spontaneous transition from E_2 to E_1 states (Kaplan 1985). Every day, kidney cells use over 2 kg of ATP to accomplish this process, and over 600g of Na^+ is reabsorbed (Feraille and Doucet 2001).

It is possible to pharmacologically inhibit the activity of NKA with specific compounds. Vanadate and digitalis glycosides, such as ouabain and digoxin, are competitive inhibitors of NKA (Cantley *et al.* 1977; Matsui and Schwartz 1968). In addition, the toxin palytoxin inhibits NKA activity by transforming the pump into a Na^+ channel, which increases intracellular Na^+ concentration (Habermann 1989).

1.4 NKA regulation in the CCD

The activity of NKA in the CCD is well regulated by many hormones and mediators, including aldosterone, AVP, insulin, FXYD proteins, substrate concentrations, dopamine, α_2-adrenergic agonist and prostaglandins. Aldosterone stimulation rapidly induces the transcription of α and β-subunit mRNA and increases NKA activity in the CCD (Verrey *et al.* 1987; Verrey, Kraehenbuhl, Rossier 1989; Welling *et al.* 1993). Similar observations were made for AVP stimulation in the CCD (Feraille and Doucet 2001; Feraille *et al.* 2003). Insulin also increases NKA activity in the CCD. This stimulation is thought to result from an increase in NKA turnover rate (Feraille *et al.* 1995). Moreover, a member of the FXYD family, FXYD4 (also named CHIF) was recently described as a regulator of NKA in the CCD (Shi *et al.* 2001). This protein can associate with the α/β complex, to increase the affinity of NKA for intracellular Na^+ (Garty *et al.* 2002). In addition, an increase in the concentration of NKA substrates (intracellular Na^+ and ATP, and extracellular K^+)

seems to induce recruitment of NKA to the cell membrane (Vinciguerra *et al.* 2003). However, the mechanisms underlying this effect remain unclear.

Furthermore, some mediators have negative effects on NKA activity. The neurotransmitter dopamine, for example, decreases NKA activity and expression in rat CCD and MDCK cells (Shahedi *et al.* 1995; Takemoto *et al.* 1992). In addition, α_2-adrenergic agonists such as epinephrine and clonidine can antagonize vasopressin-induced stimulation of Na^+ reabsorption in the CCD (Chen, Reif, Schafer 1991; Hawk and Schafer 1993; Hawk *et al.* 1993).

Another important regulator of NKA activity and the principal interest of this study is prostaglandin. The effects of prostaglandins on NKA activity and their mechanisms of action are complex, and will be described in detail in the next sections.

1.5 Prostaglandins

Prostaglandins are lipid mediators involved in many physiological processes, including inflammation, hypertension, diabetes and cancer (Cherney *et al.* 2008; Dubois *et al.* 1998; Wang and Dubois 2010). In the kidney, a major site of prostaglandin synthesis, they regulate renal blood flow, glomerular filtration rate, erythropoietin production and membrane transport (Anderson *et al.* 1976; Lote and Haylor 1989). Prostaglandins are members of the eicosanoid family. Together with thromboxane and prostacyclin (PGI_2), they form the prostanoid subclass. Prostanoids are derived from arachidonic acid, an essential fatty acid mainly found

within the cell membrane, esterified to lipids. In response to a stimulus, the cell membrane's glycerophospholipids are hydrolyzed by phospholipase A_2 (PLA_2), releasing free arachidonic acid in the cell. This fatty acid is then converted by the enzyme cyclooxygenase (COX) into prostaglandin endoperoxide H_2 (PGH_2) **(Figure 1)**.

There are two COX isoforms named COX-1 and COX-2. These enzymes exhibit similar enzymatic properties but they are derived from different genes and they differ in their regulation. COX-1 is constitutively expressed in almost all tissues, and is responsible for several housekeeping functions such as maintaining the integrity of the gastric mucosa, mediating normal platelet function, and regulating renal blood flow. COX-2 has a more restricted tissue expression under basal conditions but is rapidly induced by stress stimuli. Interestingly, studies have shown that COX-2 can also serve housekeeping functions. For example, COX-2 plays a critical role in kidney development (Komhoff *et al.* 2000). The COX enzymes contain two active sites. In the first site, there is oxygenation of the arachidonic acid to produce PGG_2, an unstable intermediate. The second site possesses a peroxidase activity that allows the conversion of PGG_2 into PGH_2, a more stable intermediate. Activity of COX-1 and COX-2 can be inhibited by non-steroidal anti-inflammatory drugs (NSAIDs), such as ibuprofen or indomethacin (Vane 2000).

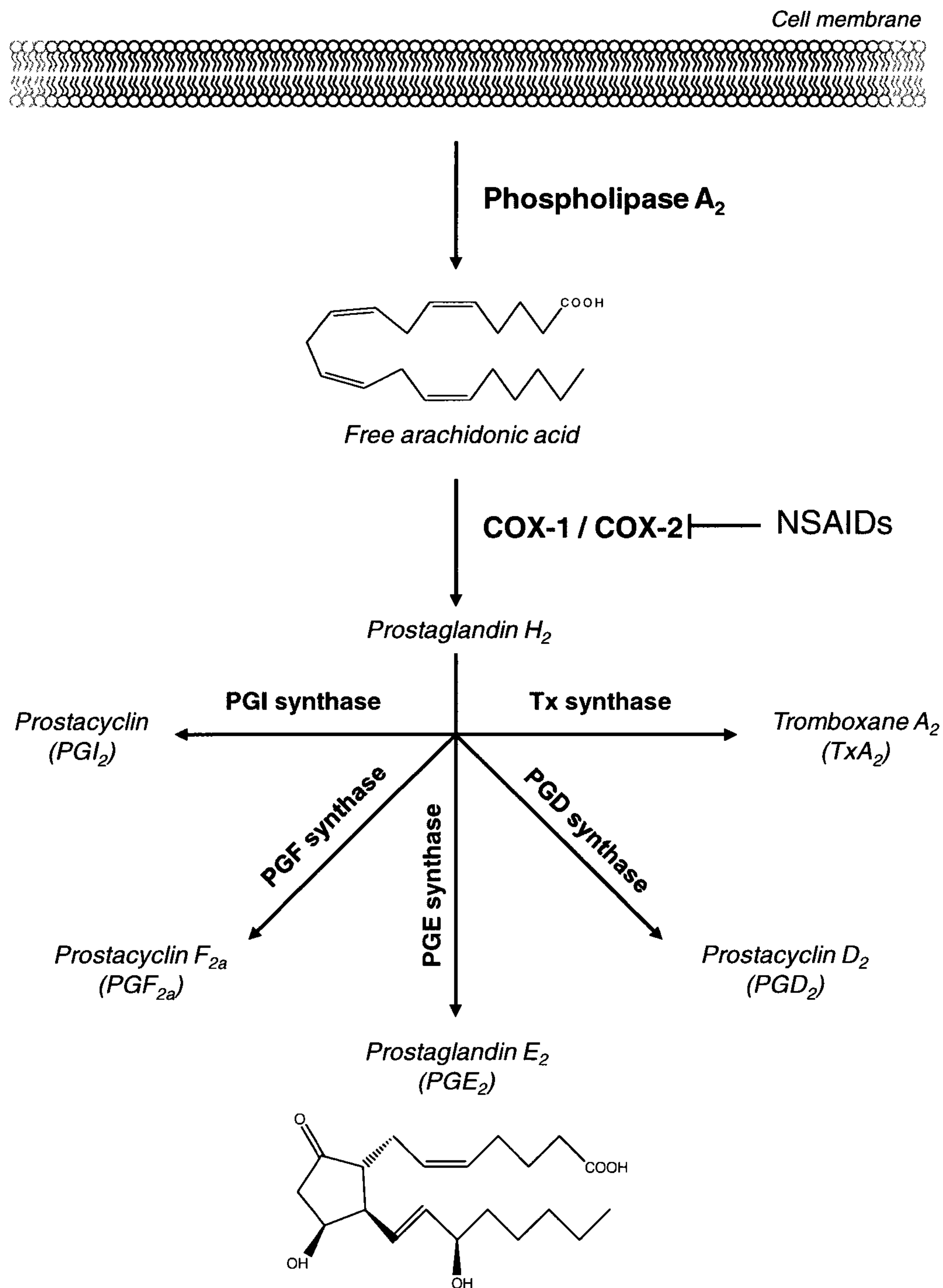


Figure 1 | Prostaglandin production by cyclooxygenases. Arachidonic acid is bound to the cell membrane, and released inside the cell by the action of phospholipase A_2. Cyclooxygenases (COX-1 and COX-2) can be inhibited by non-steroidal anti-inflammatory drugs (NSAIDs). The different enzymes participating in prostaglandin synthesis are indicated in bold.

Finally, PGH_2 is metabolized into different prostanoids including PGI_2, TxA_2, PGF_{2a}, PGD_2, PGE_2, under the action of their specific synthases (Hao and Breyer 2008; Smith, DeWitt, Garavito 2000) (**Figure 1**). PGE_2 is the most abundant prostanoid found in the kidney, and it is highly produced in the collecting duct (Bonvalet, Pradelles, Farman 1987; Larsson and Anggard 1973). Following its synthesis, PGE_2 is thought to passively diffuse outside the cell to act on resident and neighboring cells (Smith 1992). However, recent studies indicate the poor membrane permeability of PGE_2 and propose the presence of a prostaglandin efflux transporter. The multidrug resistance protein MRP4 has been identified as a potential PGE_2 transporter (Reid *et al.* 2003). Another prostaglandin transporter named PGT has been characterized at the apical membrane of CCD principal cells (Bao *et al.* 2002). It remains unclear whether this transporter mediates the release of PGE_2 to the apical side, but interesting studies have reported its role in PGE_2 reuptake by the cell (Endo *et al.* 2002; Nomura *et al.* 2004; Nomura *et al.* 2005). In fact, reuptake by PGT allows redistribution of PGE_2 to the basolateral side, where the EP receptors are mainly expressed. PGT reuptake can also lead to oxidation of PGE_2 by the 15-hydroxyprostaglandin dehydrogenase to the inactive metabolite 13,14-dihydro-15-keto PGE_2 (Hamberg and Samuelsson 1971).

1.6 Prostaglandin signaling

Following its release from the cell, PGE_2 acts as a paracrine or autocrine signaling molecule through specific G-protein-coupled receptors (GPCR) named E-prostanoid receptor subtypes (EP_1, EP_2, EP_3 and EP_4) (Hao and Breyer 2008). In

addition, there are two alternative splice variants of the EP_1 receptor and eight variants of EP_3 (Bonvalet, Pradelles, Farman 1987). GPCRs are composed of seven transmembrane domains, with an intracellular C-terminal end and an extracellular N-terminal end and they are linked to a specific G protein. EP receptors exhibit tissue specific expression and localization. The principal cells of the cortical collecting duct only express the EP_1, EP_3 and EP_4 receptor subtypes under normal conditions (**Figure 2**) (Nasrallah *et al.* 2001). All the receptors are expressed at the basolateral membrane, but the EP_4 subtype is also present on the apical side (Breyer *et al.* 1998). Binding of PGE_2 to EP_1 activates a $G\alpha_q$ protein which in turn activates phospholipase C (PLC). This enzyme is responsible for the cleavage of phosphatidylinositiol-4,5-bisphosphate (PIP_2) into inositol-1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG). IP_3 production promotes Ca^{2+} release from the ER and DAG activates protein kinase C. Activation of EP_1 in the collecting duct is known to inhibit sodium reabsorption (Larsson and Anggard 1973). The EP_3 receptor is coupled to a $G\alpha_i$ (inhibitory) protein. Its activation by PGE_2 inhibits cAMP production by adenylate cyclase leading to an inhibition of AVP-water transport (Hébert, Regnier, Peterson 1995). Finally, activation of the $G\alpha_s$-coupled EP_4 receptor (stimulatory) directly activates adenylate cyclase, increasing cAMP production. Activation of this receptor increases water reabsorption in the collecting duct (Breyer *et al.* 1998).

In addition, EP_4 activation has been reported to stimulate an additional signaling pathway under certain conditions; the phosphoinositide-3-kinase (PI3K)–dependent pathway (Fujino, Xu, Regan 2003). Activation of PI3K leads to the

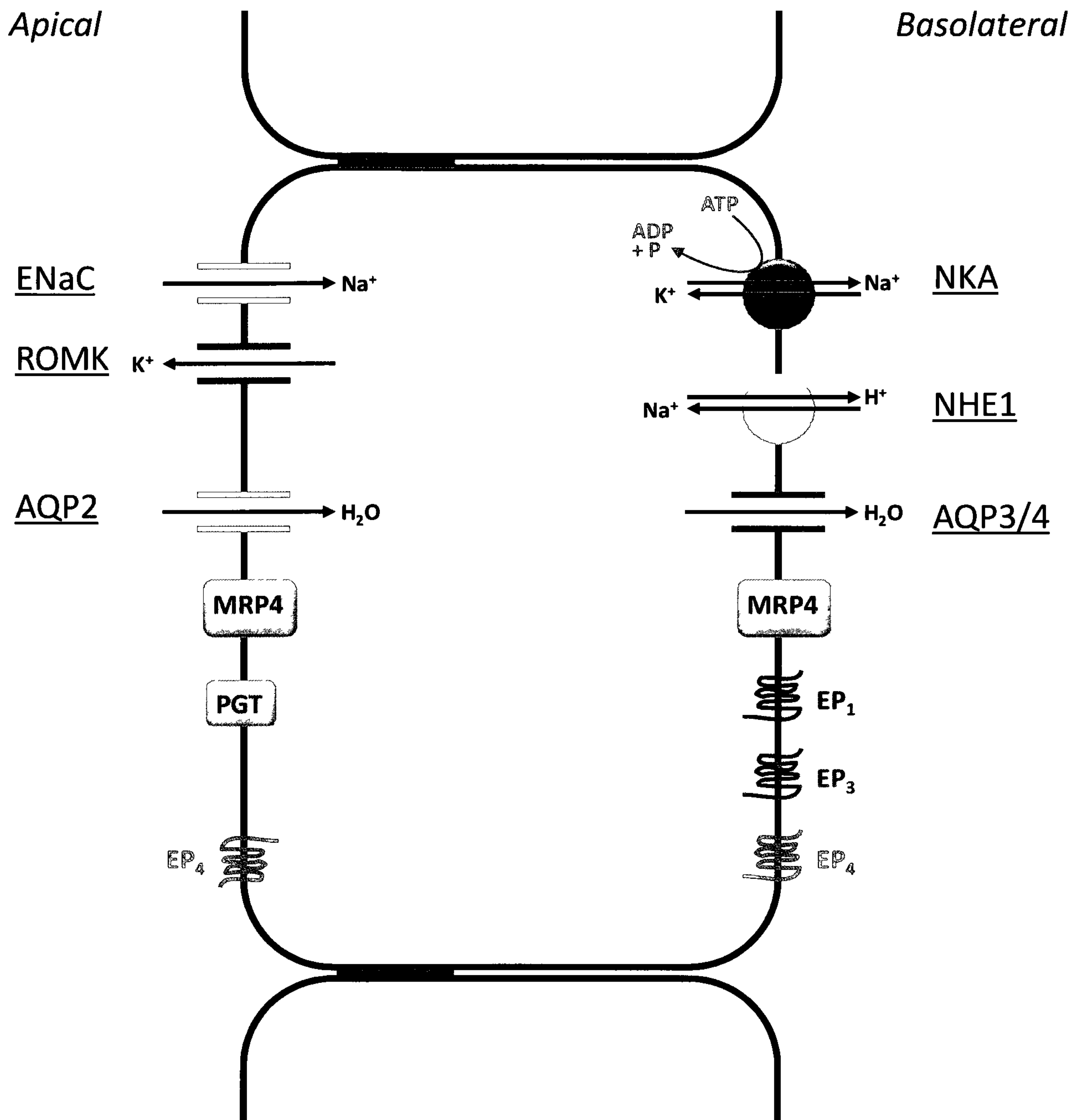


Figure 2 | Representation of salt and water transport, and PGE_2 signaling in CCD principal cells. Names of ions and water transporters (Na^+/K^+-ATPase (NKA), Na^+/H^+ exchanger (NHE1), epithelial sodium channel (ENaC), renal outer medullary potassium channel (ROMK) and aquaporins (AQP)) as well as the PGE_2 receptors (E-prostanoid receptors (EP)) and transporters (prostaglandin transporter (PGT) and multidrug resistance protein (MRP4)) are indicated. Arrows indicate the direction of Na^+, K^+ and H_2O transport.

phosphorylation of the serine-threonine kinase Akt which in turn phosphorylates many downstream effectors. The principal function of the PI3K pathway is to promote cell survival, cell growth, proliferation and differentiation (Krasilnikov 2000). This pathway can be activated by many other factors, including hormones, growth factors and cytokines (Krasilnikov 2000; Paez and Sellers 2003). These factors usually activate PI3K through receptor tyrosine kinase (RTK) or via GPCR. In that last scenario, PI3K is thought to be directly activated by the Gβγ protein complex (Schwindinger and Robishaw 2001) or indirectly by the Gα via transactivation of receptor tyrosine kinase (RTK) or integrins (Penn and Benovic 2008).

1.7 NKA regulation by PGE_2

PGE_2 plays an important role in the regulation of Na^+ and water transport in the kidney and it has been described as an important regulator of NKA along the nephron. In the proximal tubule, little is known about NKA regulation by PGE_2. A few studies have indicated a mild or no effect of PGE_2 on Na^+ transport. This can be explained by the low prostaglandin production and the low EP receptors density in this segment (Bonvalet, Pradelles, Farman 1987; Farman, Pradelles, Bonvalet 1987). However, a recent study on rabbit proximal tubule demonstrated an increase in NKA β_1-subunit transcription following stimulation with PGE_2 (Herman *et al.* 2010). They also demonstrated an increase in NKA protein levels and activity with PGE_1 treatment. In the medullary thick ascending limb of Henle's loop, an inhibition of NKA activity in the presence of PGE_2 has been reported (Rubinger *et al.* 1990; Wald *et al.* 1990). In the distal tubule, the effects of PGE_2 on Na^+ transport are unclear.

In fact, studies have reported an up-regulation of Na^+ reabsorption and NKA β_1 transcription (Matlhagela and Taub 2006; Wegmann and Nusing 2003), while others demonstrated an inhibition in NKA activity in the presence of PGE_2 in MDCK cells (Cohen-Luria, Moran, Rimon 1994). Similar observations were made in the CCD, where PGE_2 has been shown to increase (Sakairi *et al.* 1995) or inhibit Na^+ reabsorption and NKA activity (Cordova, Kokko, Marver 1989; Hébert, Jacobson, Breyer 1991; Satoh, Cohen, Katz 1992; Warden and Stokes 1993). Finally, studies have demonstrated an inhibition in NKA activity in the inner medullary collecting duct in the presence of PGE_2 (Jabs, Zeidel, Silva 1989; Zeidel, Brady, Kohan 1991).

The effects of PGE_2 on NKA modulation vary greatly along the nephron. Moreover, these regulation mechanisms can be affected by the environment surrounding the cells. All the studies mentioned above describe the action of PGE_2 in normal conditions, but little is known about the regulation of NKA by PGE_2 in hypertonic conditions. There is accumulating evidences suggesting that the PGE_2/EP receptor system is altered in hypertonic environments. First, an increase in COX-2 levels in the presence of high salt environments has been demonstrated in the cortical collecting duct (Lim *et al.* 2007), and in the medullary collecting duct (Yang *et al.* 1998). High salt treatment is also associated with an increase in prostaglandin E synthase-1 (PGES1) in the medullary region (Jia *et al.* 2006). Furthermore, it has been demonstrated that hypertonicity can modulate the expression of EP receptors in the rat kidneys (Jensen *et al.* 1999). An increase in EP_3 and EP_4 receptors has also been demonstrated in the renal medulla (Kim *et al.* 2009). However, the modulation of EP receptors depends on their localization along

the nephron and further investigations are needed to assess the expression of the receptors in the CCD under hypertonic conditions.

1.8 Cell survival/apoptosis

Hypertonic environments can be harsh for the cells and they are known to increase apoptotic events apoptosis (Kultz and Chakravarty 2001; Michea *et al.* 2000). Apoptosis refers to a specific form of programmed cell death. Under specific conditions, the cell activates death signaling pathways that will induce distinct biochemical modifications. These include a loss in cell volume, chromatine condensation, DNA fragmentation and apoptotic body formation. These modifications permit efficient phagocytosis of the cell by macrophages, without inducing an inflammatory response. Apoptosis differs from necrosis, which is an accidental death of the cell. During necrosis, cellular debris can damage other cells, and induce inflammation. Normal levels of apoptosis are good for the cell and are essential for development and homeostasis. However, too much or too less apoptosis can cause severe health conditions, such as ischemic damage, neurodegenerative disorders and cancer (Castro *et al.* 2010; Lowe and Lin 2000; Saikumar and Venkatachalam 2003).

Apoptosis of the cell occurs via two major pathways; the intrinsic and extrinsic pathways, which are respectively activated by intracellular and extracellular stimuli. Activation of the intrinsic pathway by DNA damage for example, promotes the release of pro-apoptotic factors from mitochondria in the cytoplasm. The release process is regulated by proteins of the Bcl-2 family: Bax (Bcl-2 associated X) and

Bcl-2. During apoptosis, the pro-apoptotic protein Bax is up-regulated and replaces the anti-apoptotic protein Bcl-2. The pro-apoptotic factors released in the cytoplasm, such as cytochrome c, activate specific cysteine proteases named caspases who catalyze the apoptotic response. The second pathway, the extrinsic pathway, is activated by different extracellular factors including the Fas ligand who directly binds the death receptor. Activation of this receptor induces caspases activity and promotes apoptosis of the cell. There are two types of caspases; the initiator and the effector capsases. Measuring the amount of effector caspases, such as caspase-3 is a good indication of apoptosis levels.

Apoptosis can be induced by different factors, including toxins, hormones, growth factors, cytokines, nitric oxide, pharmaceuticals at toxic doses, radiations, heat, viral infections, hypertonicity, and many more. Kidney cells, especially in the medullary region, are often exposed to hypertonic environments. High concentrations of Na^+ and urea are necessary for the counter-current mechanism to concentrate urine. Due to large fluctuations in daily dietary salt intake, CCD cells can also be exposed to hypertonic environments. Most cells would die in this highly concentrated environment, but kidney cells developed efficient regulatory mechanisms to maintain their cell volume and normal cellular functions. Interestingly, studies have suggested the action of PGE_2 on cell survival of glomerular epithelial cells via the EP_4 receptor (Aoudjit, Potapov, Takano 2006). And PGE_2 has been shown to promote cell survival via the EP_4 receptor in Jurkat cells, via a PI3K-dependent pathway (George *et al.* 2007). Furthermore, inhibition of COX by NSAIDs in renal medulla increases apoptosis following hypertonic treatment

(Hao *et al.* 2002). However, more studies are needed to determine if the PGE_2/EP receptor pathway promotes cell survival in hypertonicity in the CCD.

1.9 Rationale

The biological effects of PGE_2/EP receptors on the regulation of salt and water transport are complex as they can vary depending on the environment to which the cells are exposed. In fact, it was shown that a high salt diet can modulate EP receptor expression in the rat kidneys (Jensen *et al.* 1999). However, in the CCD, the relative expression of the different receptors in response to a high salt environment is not entirely known. Furthermore, Lim *et al.* (2007) have demonstrated that COX-2 expression is increased in a high NaCl environment in M-1 cells, a mouse CCD cell line (Lim *et al.* 2007). This increase in COX-2 suggests an alteration in prostaglandin synthesis and a possible up-regulation in PGE_2 production. We would like to determine if this modulation in prostaglandin synthesis in high salt can regulate NKA steady state levels of proteins in the CCD. Moreover, our lab has previously observed an increase in NKA α_1-subunit protein levels in high NaCl conditions *(unpublished data, honour's project, 2008)*. Preliminary analyses with EP receptor antagonists appear to reverse the high salt-induced NKA expression. However, the major prostaglandin produced, the main receptor involved and the specific signaling mechanisms implicated in this high salt modulation in the CCD are yet to be identified.

Furthermore, preliminary analyses reveal an increase in cell death and an inhibition of growth in the presence of a high NaCl environment. Similar

observations were made with renal medullary cells, where high salt treatment causes DNA damage, cell cycle arrest and apoptosis (Kultz and Chakravarty 2001; Michea *et al.* 2000). In addition, a further increase in cell loss following treatment with the specific EP_4 receptor antagonist L-161 982 in high salt was observed in our preliminary experiments. These observations correlate with recent findings suggesting that PGE_2 promotes cell survival of glomerular epithelial cells via the EP_4 receptor (Aoudjit, Potapov, Takano 2006). However, the presence of a cell survival pathway activated by the PGE_2/EP_4 receptor in the CCD has not yet been described.

A better understanding of the mechanisms underlying NKA activity and cell survival in hypertonic conditions might have important implications in the treatment of major health conditions such as hypertension and inflammation.

1.10 Purpose

The purpose of this study is to examine how the PGE_2/EP receptor system influences the steady state protein levels and activity of NKA and cell survival in a hypertonic environment, in the M-1 CCD cell line.

1.11 Hypothesis

The two hypotheses of this study are:

A) The PGE_2/EP_4 system increases NKA protein levels in M-1 CCD cells in a hypertonic environment.

B) The PGE_2/EP_4 system promotes M-1 CCD cell survival in response to high salt.

1.12 Objectives

Objective 1: To determine the effects of different hyperosmotic environments on NKA protein levels and activity.

1) *Examine NKA α and β-subunits steady state levels of protein in different hyperosmotic conditions.*
2) *Measure the activity of NKA in hypertonic conditions.*

Objective 2: To determine if PGE_2 regulates high salt-induced NKA protein level and activity.

1) *Quantify the PGE_2 production in high salt.*
2) *Characterize the EP receptor mRNA expression in hypertonic conditions.*
3) *Determine the effects of NSAIDs on NKA protein levels.*
4) *Examine the effects of EP receptor antagonists on the protein levels and activity of NKA.*
5) *Investigate the PI3K pathway.*

Objective 3: To characterize cell death and the effects of EP_4 antagonism in response to high salt.

1) *Measure apoptosis (cleaved caspace-3 protein levels, caspase-3 activity, Bax/Bcl-2 ratio, Akt phosphorylation, Hoechst 33342 staining, p38 MAPK phosphorylation) and cell viability (^3H-leucine and ^3H-thymidine incorporation) in hypertonic conditions.*
2) *Determine the effects of PGE_2 and the EP_4 antagonist on apoptosis and cell viability in high salt.*

Objective 4: To determine if the high salt-induced PGE_2 production can regulate other proteins of the CCD.

1) *Determine if COX-2 protein levels are controlled by PGE_2 in high salt.*
2) *Examine if PGE_2 can regulate NHE1 protein levels in hypertonic environment.*

2. MATERIALS AND METHODS

2.1 Cell culture

The experiments were conducted in the M-1 CCD cell line (ATCC, Manassas, VA; #CRL-2038). M-1 cells are commonly used as a model of the CCD and display many important morphological and physiological characteristics of this segment. This immortalized cell line was derived by Stoos *et al.* (1991) from a mouse transgenic for the early region of Simian Virus 40, Tg(SV40E)Bri/7 (Stoos *et al.* 1991). These cells exhibit a significant transepithelial solute gradient, an amiloride-sensitive Na^+ transport system, expression of epithelial Na^+ channel (ENaC) and expression of specific CCD antigens (Stoos *et al.* 1991). However, as opposed to previous findings, M-1 cells are not responsive to AVP (Huang *et al.* 2005).

The cells were cultured in Dulbecco's modified Eagle's medium and Ham's F-12 nutrient medium (DMEM:F12 1:1, Gibco, Carlsbad, CA) containing 7.5mM glucose, 2.5mM L-glutamine, 15mM HEPES, 0.5mM sodium pyruvate and 1.2g/L sodium bicarbonate, pH 7.4. The media was supplemented with 5% fetal bovine serum (FBS) and 1% penicillin/streptomycin (Gibco, Carlsbad, CA). The cells were grown at 37°C and 5% CO_2.

To determine the effects of hypertonicity, the cell culture media was replaced with hypertonic media 24 or 48 hours before the cells reach confluence. Composition of the different hyperosmotic media is described in **Table 1**.

Media	Concentrations *(mM)*					Total osmolality *($mOsm/kgH_2O$)*
	$[Na^+]$	$[Cl^-]$	$[Choline^+]$	[Mannitol]	[Urea]	
Control	120	120	---	---	---	240
High NaCl	240	240	---	---	---	480
High choline chloride	120	240	120	---	---	480
High mannitol	120	120	---	240	---	480
High urea	120	120	---	---	240	480

Table 1 : Ion compositions of control and hyperosmotic media.

2.2 Antagonists, inhibitors and ligands

In addition to the hypertonic environments, many experiments were performed in the presence of specific antagonists and inhibitors. Indomethacin and ibuprofen, potent COX inhibitors, were purchased from Cayman (Ann Arbor, MI). The EP_1 antagonist SC 51089 and the EP_4 antagonist L-161 982 were obtained from Enzo Life Sciences International (Plymouth Meeting, PA). The PI3K inhibitor LY294002 was obtained from Cayman (Ann Arbor, MI). Prostaglandin E_2 was purchased from Cayman (Ann Arbor, MI). The phosphodiesterase inhibitor isobutylmethylxanthine (IBMX) and the adenylate cyclase activator forskolin were obtained from Sigma (St-Louis, MO). Finally, anisomycin, a potent inhibitor of protein synthesis was purchased from Sigma (St-Louis, MO).

2.3 Western blotting

Following treatments in control and hypertonic conditions, total protein isolation was performed. First, the media was removed from the plates and the cells were washed with ice-cold phosphate buffered saline (1X PBS: 137mM NaCl, 27mM KCl, 4.3mM $NaHPO_4 \bullet 7H_2O$ and 1.4mM KH_2PO_4). The cells were scraped in 5mL of ice-cold PBS 1X and centrifuged for 5 minutes at 500 x g. The cell pellet was resuspended in RIPA protein lysis buffer (1% Nonidet P-40, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS w/v), 4.5mM NaCl, 2.5mM Tris base (pH 7.4), 8μM EDTA, 0.2mM sodium phosphate (pH 7.2), 0.5mM phenylmethanesulfonyl fluoride (PMSF), 1:100 protease inhibitor cocktail (Sigma, St.Louis), 1mM sodium pyrophosphate, 10mM sodium fluoride and 100μM sodium orthovanadate) and sonicated for 5 seconds. Next, the cell lysate was centrifuged at 10 000 x g for 10 minutes at 4°C, and the supernatant was transferred to a clean Eppendorf tube.

Protein quantification was performed using Bradford reagent (Bio-Rad, Hercules, CA). Samples were combined with the Laemmli loading buffer and heated at 70°C for 15 minutes to denature the proteins. Samples were then loaded into a polyacrylamide gel (10-17% resolving gel with a 4% stacking gel) and electrophoresed at 150V for approximately 60 minutes. Next, the proteins were transferred onto a nitrocellulose membrane (GE healthcare, Piscataway, NJ) at 100V for 70 minutes. Membranes were blocked in 5-10% milk/TBS-T (137mM NaCl, 20mM Tris base, 0.1% Tween 20) for 90 minutes and incubated overnight with a primary antibody. Three washes of 15 minutes in TBS-T were performed the next

day and the appropriate secondary antibody (in 5% milk/TBS-T) was applied to the membranes for 90 minutes. Next, the membranes were washed three times in TBS-T for 90 minutes. Finally, the bands were developed on X-Omat Blue Kodak films (Perkin Elmer, Waltham, MA) using SuperSignal West Pico Chemiluminescent reagents (Pierce, Rockford, IL). A loading control was made by measuring the protein levels of β-actin or Gβ. Densitometric analysis was performed on the Alpha Innotech FluorchemHD2 imaging system. A list of antibodies with their respective dilutions and secondary antibody is provided in **Table 2**.

1° Antibody		2° Antibody	
Name	Dilution	Name	Dilution
β-actin (Sigma)	1:10000, 5% milk	Anti-mouse IgG HRP (Promega)	1:2000
β-catenin (Sigma)	1:40000, 10% milk	Anti-rabbit IgG HRP (Promega)	1:2000
Bax (Santa Cruz)	1:1000, 5% milk	Anti-mouse IgG HRP (Promega)	1:2000
Bcl-2 (Santa Cruz)	1:1000, 5% milk	Anti-mouse IgG HRP (Promega)	1:2000
Cleaved Caspase-3 (Cell signaling)	1:1000, 5% BSA	Anti-rabbit IgG HRP (Promega)	1:2000
COX-2 (Cayman)	1:4000, 10% milk	Anti-rabbit IgG HRP (Promega)	1:2000
Gβ (Santa Cruz)	1:2000, 5% milk	Anti-rabbit IgG HRP (Promega)	1:2000
Na^+/K^+-ATPase α_1 (Promega)	1:20000, 5% milk	Anti-mouse IgG HRP (Promega)	1:2000
Na^+/K^+-ATPase β-1 (Santa Cruz)	1:500, 5% milk	Anti-goat IgG HRP (Santa Cruz)	1:2000
NHE1 (Santa Cruz)	1:500, 5% milk	Anti-rabbit IgG HRP (Promega)	1:2000
Phospho-Akt (Cell signaling)	1:2000, 5% BSA	Anti-rabbit IgG HRP (Promega)	1:2000
Total Akt (Cell signaling)	1:2000, 5% BSA	Anti-rabbit IgG HRP (Promega)	1:2000
Phospho-P38 (Cell signaling)	1:1000, 5% BSA	Anti-rabbit IgG HRP (Promega)	1:2000
Total-P38 (Cell signaling)	1:1000, 5% BSA	Anti-rabbit IgG HRP (Promega)	1:2000

Table 2 : Immunoblotting antibodies and dilutions.

BSA: bovine serum albumin

2.4 NKA activity

2.4.1 Membrane preparations

Separation of membrane proteins from cytosolic proteins was performed by ultracentrifugation. First, the cells were stimulated in control or hypertonic conditions for 24 or 48 hours and washed with ice-cold PBS 1X. Then, the cells were scraped in 80 µL of lysis buffer A (50mM Tris base, 2mM Na_2EDTA, pH 7.4, 1:100 protease inhibitor cocktail, 1mM sodium pyrophosphate, 10mM sodium fluoride and 100µM sodium orthovanadate). The samples were sonicated for 5 seconds and centrifuged for 10 minutes at 3000 x g. The supernatant was transferred into an ultracentrifuge Eppendorf tube and centrifuged at 83714 x g for 1 hour at 4°C. The supernatant, representing the cytosolic fraction, was transferred into a clean Eppendorf tube, and the pellet, representing the membrane fraction, was dissolved in 20-60µL of lysis buffer B (50mM Tris base, 2mM Na_2EDTA, 300mM NaCl, 1% Triton, 0.1% SDS, pH 7.4, 1:100 protease inhibitor cocktail, 1mM sodium pyrophosphate, 10mM sodium fluoride and 100µM sodium orthovanadate). Protein levels were determined with Bradford reagent, and the western blotting protocol described in section 2.3 was performed to determine the expression of NKA α_1 in cytosol and membrane fractions.

2.4.2 Detection of free phosphate ions

To estimate the activity of NKA, the production of free phosphate ions was measured in control and hypertonic environments with the colorimetric Quantichrom™ ATPase/GTPase Assay Kit (BioAssay Systems, Hayward, CA). The cells were cultured in 100mm Petri dishes and stimulated with hypertonic media for 3, 6 and 24 hours. Total protein isolation was performed as described in section 2.3, but PBS was replaced with a Tris buffer (20mM Tris base, 0.9% NaCl, pH 7.4) and RIPA lysis buffer was changed for the one provided with the assay. Total proteins were quantified using Bradford reagent and the ATPase assay was immediately performed following manufacturer's instructions. Briefly, 0.2µg of proteins were incubated in the presence of 1mM ATP for 30 minutes. The malachite green reagent was then added to the reaction for 30 minutes. This reagent forms a stable dark green color with liberated phosphate ions. The concentration of free phosphate ions was determined by measuring the absorbance of the plate at 595nm with the FLUOstar Galaxy plate reader system (BMG Labtechnologies, Offenburg, Germany) and comparing the results to a standard curve made with unknown concentrations of phosphate. Each sample was assayed in triplicate and the results are expressed as fold control.

2.5 RNA isolation and real-time PCR

Real-time PCR was performed to determine the levels of EP receptors in control and high salt conditions. First, the cells were incubated for 24 hours in control or hypertonic media. Following manufacturer's instructions, mRNA isolation

was done using 1mL of TRIzol reagent (Gibco, Carlsbad, CA). mRNA samples were dissolved in 20μL of diethylpyrocarbonate (DEPC)-treated water and treated with 0.3 U/ug DNAse I (Invitrogen, Carlsbad, CA). Real-time PCR was then performed on 100ng of mRNA, using the TaqMan One-step RT-PCR master mix reagents (Applied Biosystems, Foster City, CA) and the ABI Prism 7000 sequence detection system. The sequences of the probes and primers are presented in **Table 3**. The amplification conditions consisted of: 30 minutes at 48°C, 10 minutes at 95°C and 40 cycles of 15 seconds at 95°C and 1 minute at 60°C. Expression of each receptor was normalized to GAPDH mRNA levels, detected with the TaqMan Rodent GAPDH control reagent kit (Applied Biosystems, Foster City, CA). Concentrations of unknown RNAs were determined with standard curve analysis of known concentrations of RNA submitted to qPCR amplification. A cycle threshold (C_t) was established for each experiments and the number of cycles at which an unknown sample reached the C_t was converted in ng of RNA. A relative quantification was performed for each receptor and results are expressed as fold control.

Receptor	Size	Primer sequences	Probes sequences
EP_1	336 bp	Sense: agtgccaagggtggtccaa	6FAM-tgggcctaaccaagagtgcctgta-TAMRA
		Anti-sense: ccgggaactacgcagtgaac	
EP_3	437 bp	Sense: gccgctattgataatgatgttgaa	6FAM-tcaatcagatgtcggttgagcaatgcaa-TAMRA
		Anti-sense: ccttctcctttcccatctgtgt	
EP_4	423 bp	Sense: atggtcatcttactcatcgccac	6FAM-catctgctccattccgctcgtggt-TAMRA
		Anti-sense: ctttcaccacgtttggctgat	

Table 3 : Primer and probe sequences for mouse EP receptors.

2.6 Enzyme immunoassay

In order to measure PGE_2 production, the cells were cultured for 24 hours in control and hypertonic media, with or without indomethacin (10μM) or ibuprofen (10μM). The media was then collected and the amount of PGE_2 was determined by competitive enzyme immunoassay (EIA, Cayman, Ann Arbor, MI), according to the manufacturer's instructions. Briefly, the assay consists of a competition between the PGE_2 produced by the cells and an acetylcholinesterase tracer, for a specific amount of monoclonal antibody. The amount of tracer bound to the antibody was detected by colorimetric reaction using Ellman's reagent and the absorbance was measured at 420nm with the FLUOstar Galaxy plate reader system. The amount of PGE_2 produced by the cells is inversely proportional to the amount of tracer detected. Each experiment was performed in triplicate and the results are expressed as fold control.

2.7 ^{3}H-cAMP assay

cAMP production was assessed to study PGE_2 signaling responses in M-1 cells. The cells were incubated for 24 hours in control or high NaCl media in 24-wells plates and serum starved for 24 hours. The cells were then pretreated for 15 minutes with DMEM:F12 containing 0.5mM IBMX and 10μM indomethacin to inhibit cAMP degradation and prostaglandin production. To determine the effect of EP_4 antagonism on cAMP production, some of the wells were pre-incubated for 10 minutes with 10μM of L-161 982. The cells were then stimulated for 10 minutes in

control or high NaCl media with 10μM forskolin, 1μM PGE_2, 10μM indomethacin and 10μM L-161 982. Addition of 300 μL of ice-cold triochloroacetic acid (10% TCA v/v) was used to stop the stimulation and the cells were incubated at 4°C for 30 minutes. Next, the samples were centrifuged at 1500 x g for 10 minutes and transferred into glass test-tubes for ether extraction of TCA. Four extractions were done using 800μL of H_2O-saturated diethyl ether each time. A competitive cAMP binding assay was then performed with the ^{3}H-cAMP DPC radioassay (GE healthcare, Piscataway, NJ) following manufacturer's instructions. Briefly, the assay consists of a competition between the cAMP produced by the cells and ^{3}H-cAMP, for a specific amount of binding proteins. After two hours of incubation with binding proteins, the free cAMP was removed with 250 μL of charcoal followed by a centrifugation at 12720 x g for 5 minutes at 4°C. The supernatant was transferred into scintillation vials containing 10 mL of scintillation fluid and ^{3}H-cAMP radioactivity was measured in disintegration per minute (dpm) using a scintillation counter. The levels of ^{3}H-cAMP detected are inversely proportional to the amount of cAMP produced by the cells. Each experiment was performed in duplicate and cAMP production was calculated in percent stimulation of the control.

2.8 Measurement of intracellular Ca^{2+}

Intracellular Ca^{2+} was measured as described by He *et al.* (2005), to investigate the EP_1 signaling pathway (He *et al.* 2005). Cells were grown to confluence in 35 mm Petri dishes on 30 mm glass coverslips in control media. The cells were washed with PBS 1X and incubated for 10 minutes at 37°C in the

presence of the selective fluorescent probe Fura-2AM (4µM in 0.01% pluronic; Invitrogen, Carlsbad,CA). Then, the coverslips were placed in a temperature-regulated chamber (37°C) mounted on the stage of an inverted microscope. The chamber was continuously perfused (2mL/min) with Hanks' buffer (137mM NaCl, 4.2mM $NaHCO_3$, 3mM Na_2HPO_4, 5.4mM KCl, 0.4mM KH_2PO_4, 1.3mM $CaCl_2$, 0.5mM $MgCl_2$, 0.8mM $MgSO_4$, 5.5mM glucose, 5mM HEPES, pH 7.4). Cells were stimulated with 1µM PGE_2 and 1µM aldosterone and the Ca^{2+} concentration was measured in multiple cells simultaneously by the Stallion Digital Hi-Speed Multi-Channel Imaging System (Attofluor Ratiovision, Zeiss, Germany), using an emission wavelength of 520 nm and alternating excitatory wavelengths of 340 nm and 380 nm. As a positive control, the cells were incubated in the presence of ionomycin (1µM, Sigma, St-Louis, MO), a potent and selective Ca^{2+} ionophore agent. Results are expressed as 340/380 nm ratio of three region of interest randomly selected per stimulation.

2.9 Fluorescent caspase-3 activity assay

The activity of caspase-3 was measured to evaluate the effect of hypertonicity and EP_4 antagonism on cell death with the CASPASE-3 Cellular Activity Assay Kit PLUS (BIOMOL, Plymouth meeting, PA). The cells were grown in 100mm Petri dishes for 24 hours with control or high NaCl media, in the presence of 10µM indomethacin, 10µM L-161 982 or 1µM PGE_2. The media was removed and the cells were washed with 1X ice-cold PBS. The cells were scraped in 5mL of ice-cold PBS and centrifuged for 2 minutes at 500 x g. The pellet was resuspended in 100µL of

cell lysis buffer provided in the kit. To disrupt the cell membrane, two freeze-thaw cycles at -20°C were performed and the samples were incubated on ice for 15 minutes. Then, the samples were centrifuged at 10 000 x g for 10 minutes at 4°C, and the supernatant was collected in a clean Eppendorf tube. Total protein concentration was determined using Bradford reagent. After that, the caspase-3 activity assay was performed, according to manufacturer's instructions. Briefly, 50µg of proteins were loaded in duplicate on a 96-well plate. Then, 0.4µM of the caspase inhibitor Ac-DEVD-CHO was added into one of the replicate wells, and 20µM of the substrate Ac-DEVD-AMC was added to all the wells. The plate was incubated at room temperature for 24 hours and the fluorescence intensity was detected with the FLUOstar Galaxy plate reader, with an excitation wavelength of 360 nm and an emission wavelength of 460 nm. Results are expressed as fold control.

2.10 Hoechst 33342 staining

To illustrate the effect of hypertonicity and L-161 982 on cell viability, the cells were stained with Hoechst 33342. First, the cells were cultured for 24 hours in control or hypertonic environments, with indomethacin (10 µM), PGE_2 (1 µM) or L-161 982 (10 µM) on 15mm glass coverslips in a 12-well plate. Media was then removed and the cells were fixed for 20 minutes with 4% paraformaldehyde. The wells were washed two times with PBS and exposed to Hoechst 33342 dye (10µg/ml, Invitrogen, Carlsbad,CA) for 30 minutes. The coverslips were mounted on glass slides and evaluated under the fluorescent microscope Axioskop Mot 2 (Zeiss,

Jena, Germany) with the DAPI filter (excitation wavelength of 360nm and emission wavelength of 450nm). Images were captured with the camera AxioCam (Zeiss, Jena, Germany) and number of cells with apoptotic morphology was counted in two random fields for each coverslip. Quantitative analysis of the cells was performed in a blinded fashion relative to the different treatments. Each treatment was performed in duplicate (two coverslips per treatment) and the results are expressed as percentage of apoptotic nuclei compared to the total number of cells.

2.11 ^{3}H-thymidine incorporation

DNA synthesis was measured using ^{3}H-thymidine incorporation to determine the effect of hypertonicity and EP_4 antagonism on cell growth. M-1 cells were grown on 24 well-plates and starved 24 hours prior to stimulation. Then, the cells were stimulated for 24 hours in control or high NaCl media with 1µM PGE_2, 10µM indomethacin and 10µM L-161 982. An hour before the end of the stimulation, 0.5µCi of ^{3}H-thymidine (Perkin Elmer, Waltham, MA) was added to the wells. The plates were then washed four times in ice-cold PBS 1X and permeabilized in 500µL of NaOH 1N at 37°C for 30 minutes. Next, the cells were transferred into scintillation vials containing 10mL of scintillation fluid and ^{3}H-thymidine quantity was measured in dpm using a scintillation counter. Each experiment was done in triplicate and thymidine incorporation is expressed as fold control.

2.12 ^{3}H-leucine incorporation

Protein synthesis was measured to determine the effect of hypertonicity and EP_4 antagonism on cell growth. M-1 cells were grown on 24 well-plates and starved 24 hours prior to stimulation. Then, the cells were stimulated for 24 hours in control or high NaCl media with 1μM PGE_2, 10μM indomethacin and 10μM L-161 982. In addition, 0.5μCi of ^{3}H-leucine (Perkin Elmer, Waltham, MA) was added to the wells for the 24 hours of stimulation. The plates were then washed four times in ice-cold PBS 1X and permeabilized in 500μL of NaOH 1N at 37°C for 30 minutes. Next, the cells were transferred into scintillation vials containing 10mL of scintillation fluid and the quantity of ^{3}H-leucine was measured in dpm using a scintillation counter. Each treatment was done in triplicate and leucine incorporation is expressed as fold control.

2.13 Statistics

Graphpad Prism (version 5.03) was used to analyze and present the data. Values are expressed as means ± standard error of the mean (SEM). Statistical analysis for multiple comparisons was done using one-way ANOVA corrected with Bonferroni's post test. A $p\text{-value} < 0.05$ with $n \geq 3$ was considered statistically significant.

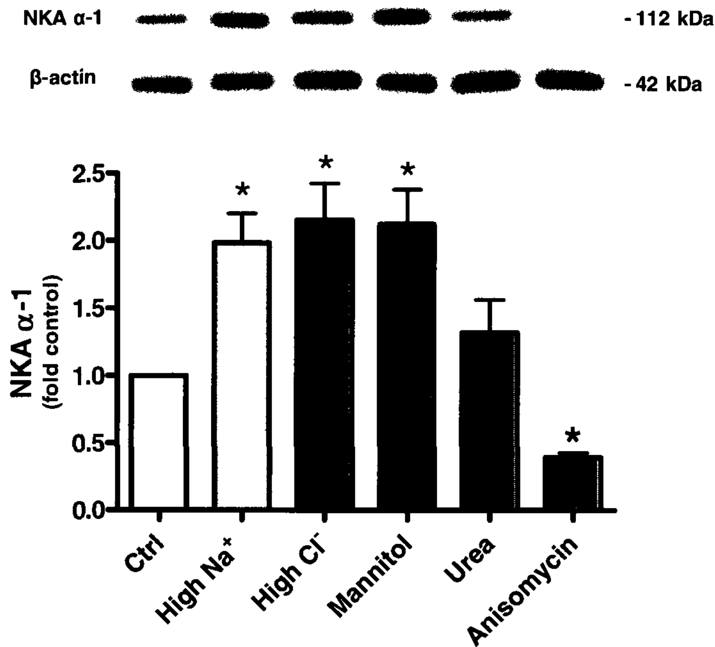
3. RESULTS

3.1 Hypertonicity increases NKA α_1 protein levels.

To determine the effect of hypertonicity on the protein levels of NKA in the CCD, M-1 cells were incubated in a control media containing 120mM of NaCl (240 mOsm/kgH_2O) or in the presence of four different hyperosmotic environments (480mOsm/kgH_2O): high NaCl, high choline chloride, high mannitol and high urea. High choline chloride was used as a control to determine the influence of the Cl^- ion. Mannitol was used to determine if hypertonicity rather than NaCl only was responsible for the changes in NKA. Finally, the isotonic hyperosmotic environment created with urea was used to determine if hyperosmolality or hypertonicity was involved in NKA modulation. After 24 and 48 hours of incubation in hypertonic conditions, a 2-fold increase in NKA α_1-subunit steady state levels of protein was detected by Western blotting (**Figure 3**). This increase in NKA α_1 was not detected in the presence of urea suggesting that hypertonicity and not hyperosmolality is causing the change in NKA levels. Treatment with anisomycin (150ng/mL), a potent protein synthesis inhibitor, was also performed to determine if the increase in NKA protein level in hypertonic conditions is a result of cell apoptosis. As illustrated in **Figure 3**, anisomycin reduced considerably the levels of NKA α_1.

The protein levels of β_1-subunit were also measured by Western blotting. As shown in **Figure 4**, no change was detected in β_1 levels in hypertonic environments after 24 or 48 hours. However, anisomycin treatment reduced significantly the protein levels of NKA β_1.

A | 24 hours



B | 48 hours

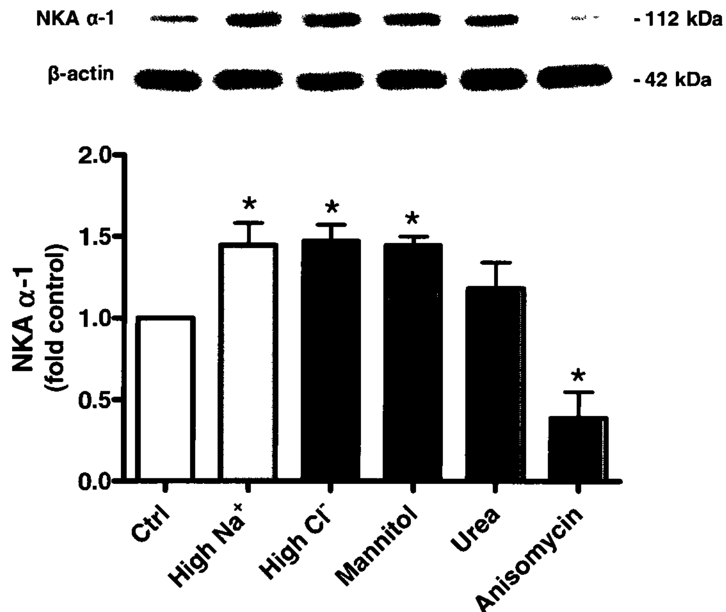
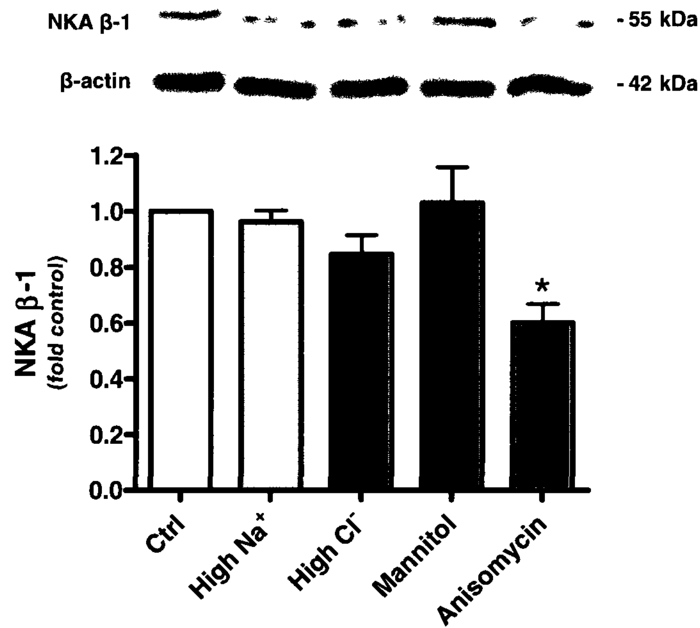


Figure 3 | Protein levels of NKA α_1-subunit are increased in hypertonic conditions and reduced by anisomycin treatment. M-1 cells were incubated in control (120mM NaCl) or hyperosmotic media (NaCl, choline chloride, mannitol or urea 240 mM). The cells were also treated with anisomycin 150ng/ml. Total protein was analyzed by immunoblotting to examine the expression of NKA α_1-subunit after **A)** 24 hours (n=6 to 12), and **B)** 48 hours (n= 4 to 7). Expression of NKA α_1 was quantified by densitometry, adjusted for β-actin and expressed as fold control with ctrl=1. ANOVA, mean ± SEM * = $p < 0.05$

A|24 hours



B|48 hours

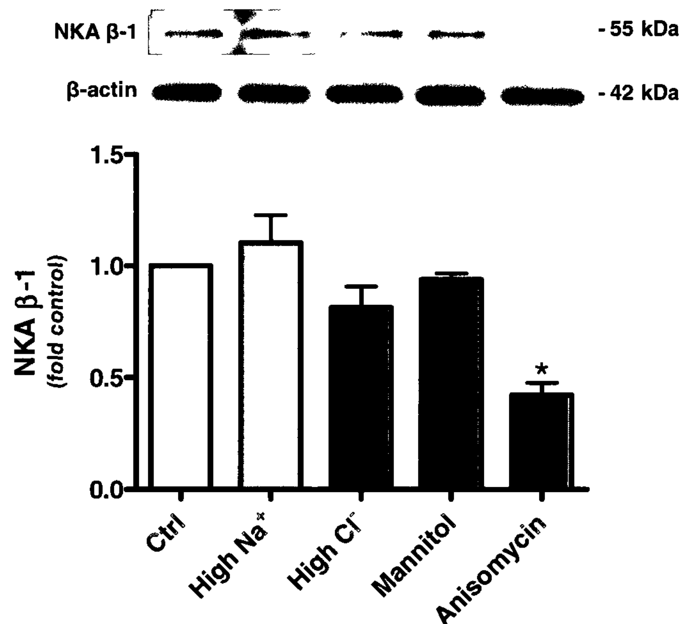
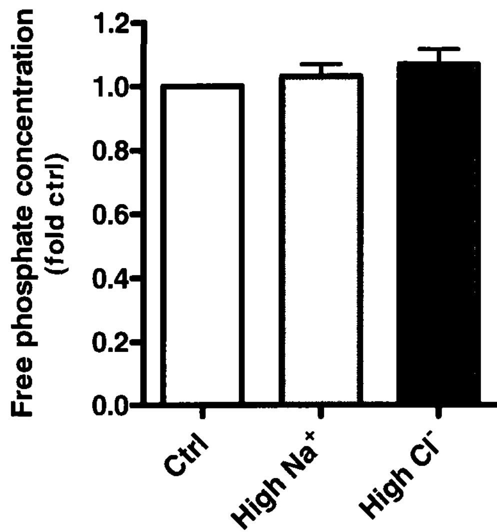


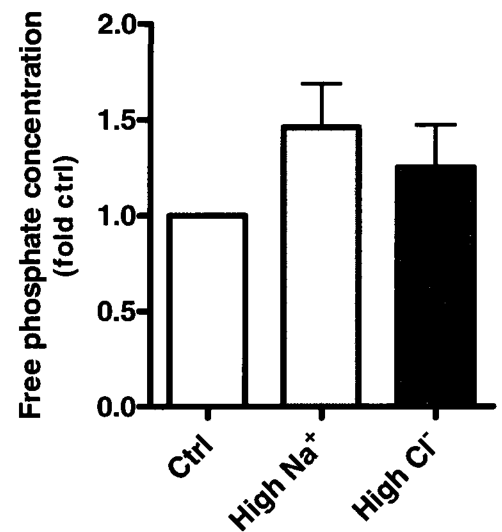
Figure 4 | Protein levels of NKA β_1-subunit are not affected by hypertonic conditions but reduced by anisomycin treatment. M-1 cells were incubated in control (120mM NaCl) or hyperosmotic media (NaCl, choline chloride or mannitol 240 mM). The cells were also treated with anisomycin 150ng/ml. Total protein was analyzed by immunoblotting to examine the expression of NKA β_1-subunit after **A)** 24 hours (n=3 to 7), and **B)** 48 hours (n= 3 to 5). Expression of NKA β_1 was quantified by densitometry, adjusted for β-actin and expressed as fold control with ctrl=1. ANOVA, mean ± SEM * = $p < 0.01$

3.2 Hypertonicity increases NKA α_1 membrane protein expression.

The activity of NKA in hypertonic environments was also investigated. In a first attempt, the production of free phosphate ions was measured with the colorimetric QuantiChrom™ ATPase/GTPase Assay Kit. No change in free phosphate ion production was detected after 3, 6 and 24 hours of incubation in high salt, suggesting no change in the total ATPase activity in the cell (**Figure 5**). However, free phosphate ions are not only produced by NKA, and this assay is not sufficient to conclude that NKA activity is not affected by high salt treatment.

Another way to estimate NKA activity in the cell is to quantify its protein expression at the cell surface. Cytosolic and membrane proteins were separated by ultra-centrifugation and both fractions were analyzed by immunoblotting. NKA α_1-subunit was increased by 2.5 fold after 24 and 48 hours of incubation in hypertonic conditions (**Figure 6A and C**). A similar increase was observed in the cytosol (**Figure 6 B and D**). As an indicator of the success of the protein separation procedure, detection of β-actin (a cytoplasmic protein) in the membrane fraction was not possible. Instead, the levels of the Gβ protein was used as a loading control for the membrane fraction.





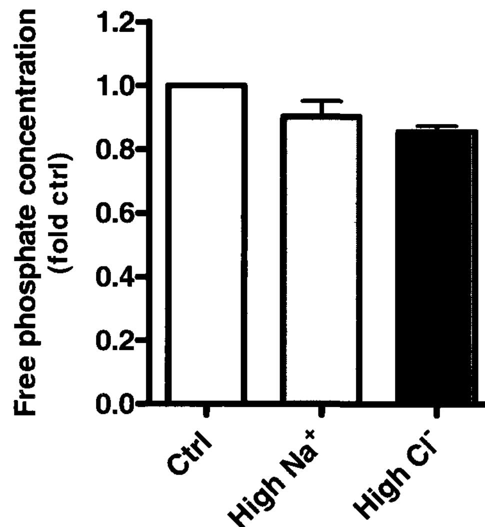
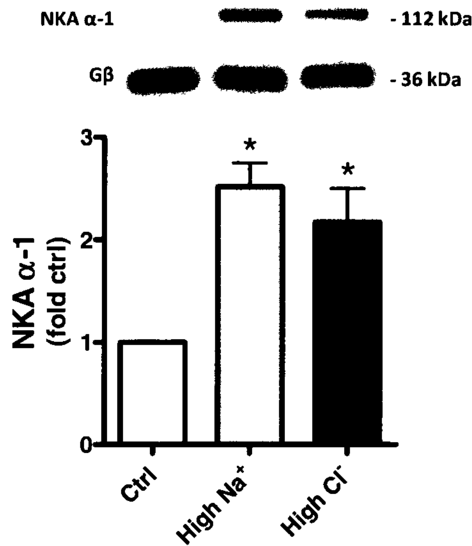
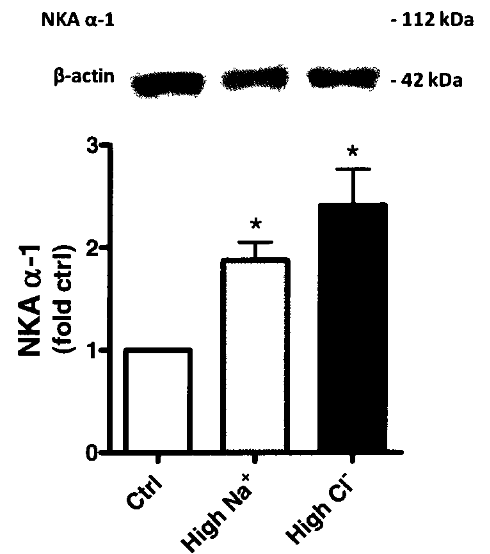
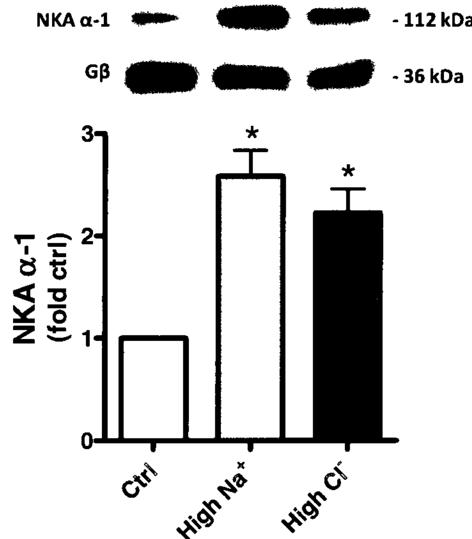


Figure 5 | Free phosphate ion production is not increased by hypertonicity. M-1 cells were incubated in control (120mM NaCl) or hyperosmotic media (NaCl or choline chloride, 240 mM) and total proteins were isolated and quantified. NKA activity was measured using the QuantiChrom™ ATPase/GTPase Assay kit after **A)** 3 hours (n=3), **B)** 6 hours (n=3 to 5) and **C)** 24 hours (n=3 to 5). The amount of free phosphate produced from ATP hydrolysis was determined by measuring the absorbance at 595nm. The relative amounts of free phosphate were compared to the control and expressed as fold control with ctrl=1.







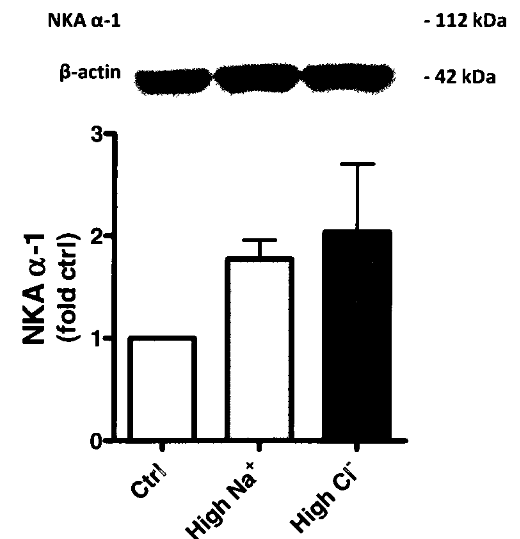


Figure 6 | Protein levels of NKA α_1-subunit are increased in hypertonic conditions at the cell membrane and in the cytosol. M-1 cells were incubated in control (120mM NaCl) or hypertonic media (NaCl or choline chloride, 240 mM) for 24 and 48 hours. Cytosolic proteins were separated from cell membrane proteins by ultracentrifugation, and analyzed by immunoblotting to examine the expression of NKA α_1-subunit. **A)** Membrane proteins after 24 hours (n=6), **B)** cytosolic proteins after 24 hours (n=4), **C)** membrane proteins after 48 hours (n=5) and **D)** cytosolic proteins after 48 hours (n=3). Expression of NKA α_1 was quantified by densitometry, adjusted for β-actin in the cytosol, and Gβ at the cell membrane and expressed as fold control with ctrl=1. ANOVA, mean ± SEM * = $p < 0.05$

3.3 PGE_2 production is increased by hypertonicity.

In order to determine if PGE_2 is involved in NKA regulation, its expression was assessed in control and hypertonic conditions. Our lab previously confirmed an increase in COX-2 protein steady state levels in a high salt environment in M-1 cells *(unpublished data, honour's project, 2008)*, suggesting an increase in prostaglandin synthesis. The next step was to quantify the production of PGE_2 by enzyme immunoassay (EIA). As illustrated in **Figure 7**, PGE_2 was increased by 3.5 fold after 24 hours of incubation in high NaCl conditions. Furthermore, the salt-induced increase in PGE_2 was completely inhibited by pre-treatment with indomethacin or ibuprofen in a high NaCl environment.

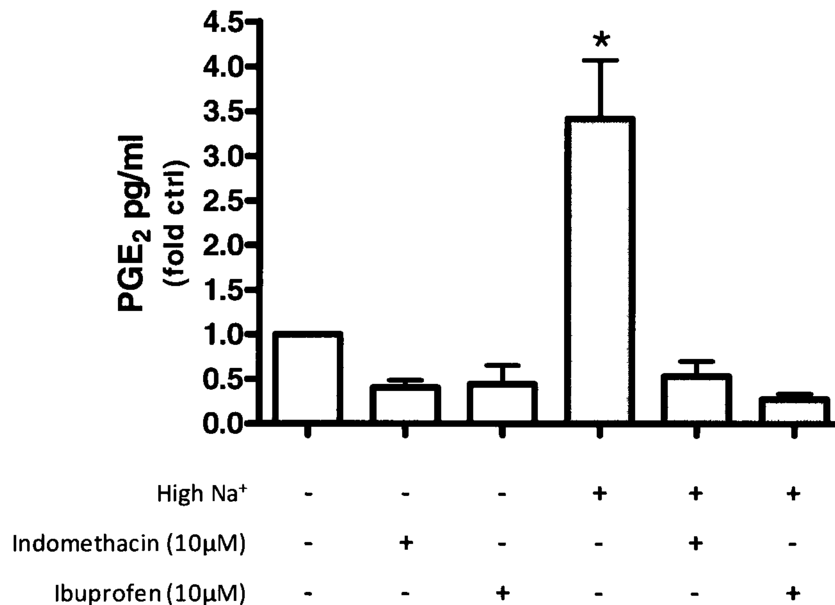


Figure 7 | PGE_2 production is increased in a hypertonic environment and reversed by NSAIDs. M-1 cells were incubated in control (120mM NaCl) or high NaCl media (240 mM NaCl) with indomethacin (10µM) or ibuprofen (10µM) for 24 hours. The media was collected after 24 hours to measure PGE_2 (n=3 to 5) by enzyme immunoassay (EIA). PGE_2 levels are expressed in pg/mL as fold control with ctrl=1. ANOVA, mean ± SEM * = $p < 0.01$.

3.4 EP receptor expression is altered in hypertonic conditions.

The PGE_2 signaling pathway does not only depend on PGE_2 production. It can also be regulated at the receptor level, by increasing or reducing the expression of the multiple EP receptors. The relative mRNA expression of EP_1, EP_3 and EP_4 receptors in high salt was determined by real-time PCR. As shown in **Figure 8**, the mRNA levels of EP_1 and EP_4 remain constant in hypertonic environments, while EP_3 mRNA is increased up to 9 fold by high Na^+, and up to 6 fold with Cl^- alone. In addition, expression of EP_1 receptor mRNA was higher than EP_3 and EP_4 in M-1 cells in normal and hypertonic conditions, since a smaller amount of RNA was used (50ng), and EP_1 amplified faster than the other EP receptors.

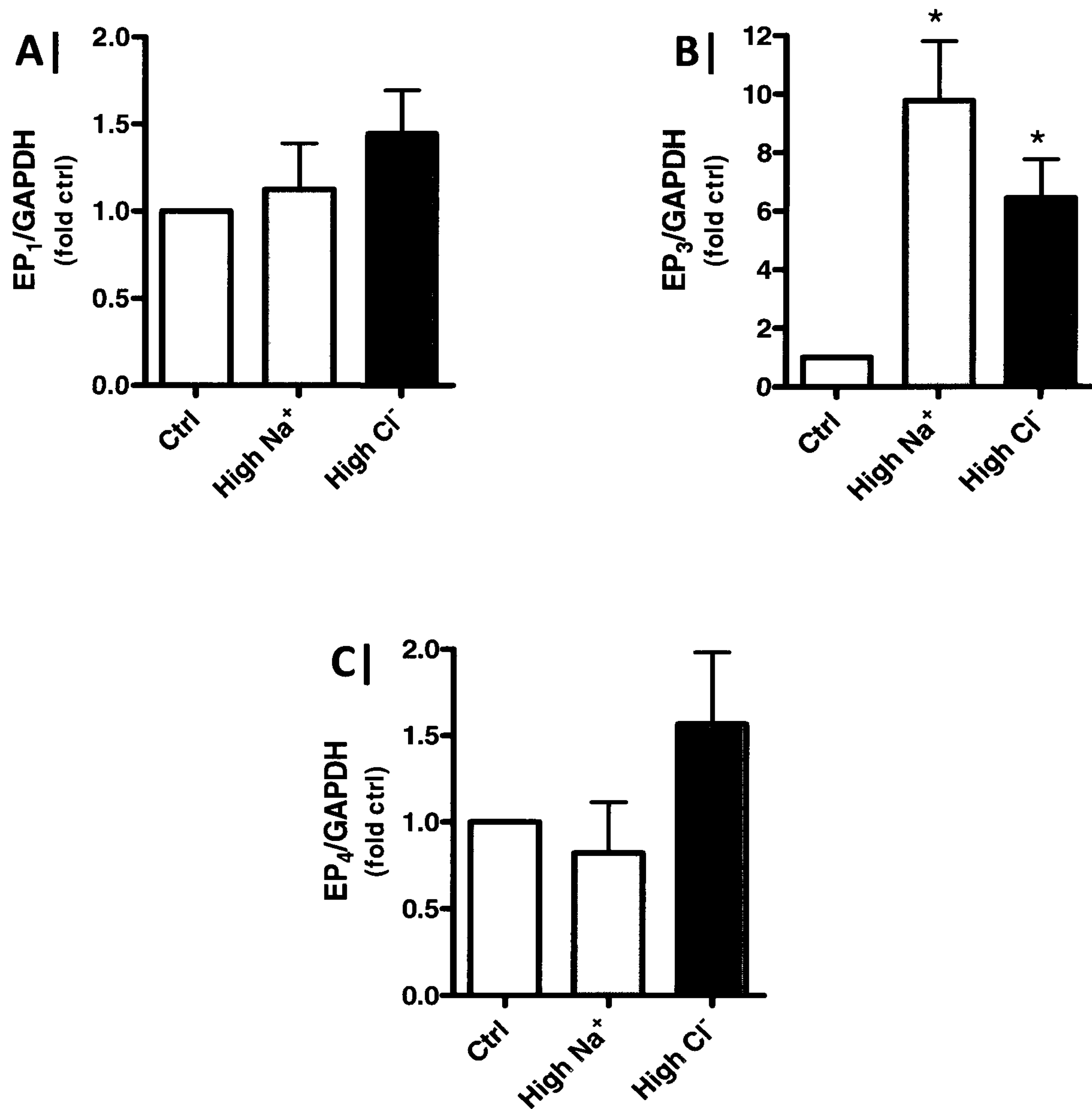


Figure 8 | EP_1, EP_3 and EP_4 mRNA expression is altered in hypertonic conditions. M-1 cells were incubated in control (120mM NaCl) or hypertonic media (NaCl or choline chloride, 240 mM) for 24 hours. Total mRNA was isolated with Trizol reagent and mRNA expression for **A)** EP_1 receptor (n=7), **B)** EP_3 receptor (n=5) and **C)** EP_4 receptor (n=5) was analyzed by real-time PCR, normalized to GAPDH and expressed as fold control with ctrl=1. ANOVA, mean ± SEM * = $p < 0.05$.

3.5 EP_4 antagonism reduces NKA protein levels.

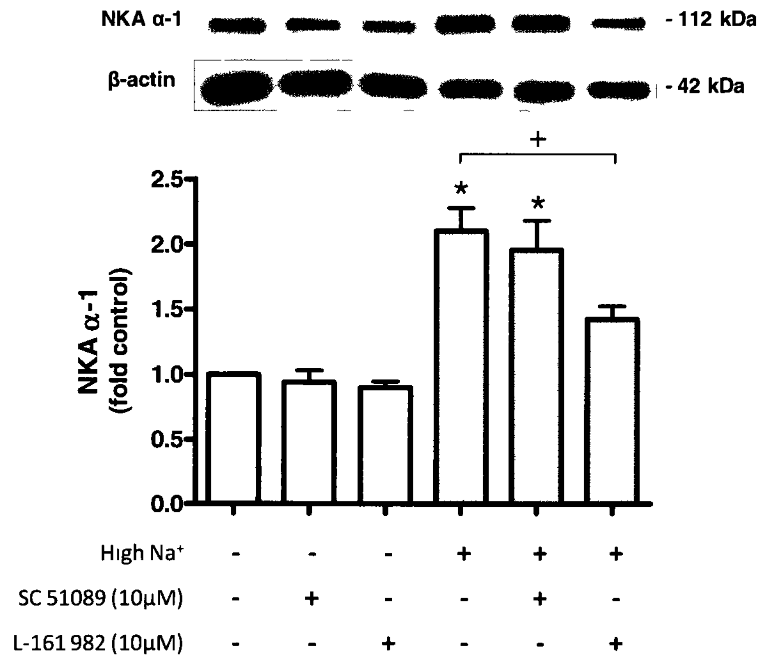
Our first step to determine the role of the PGE_2/EP receptor system on NKA regulation in high salt was to block the EP receptors with specific EP antagonists in hypertonic conditions. Following treatment, total proteins were isolated and the protein levels of NKA α_1 was analyzed by immunoblotting. As illustrated in **Figure 9**, levels of NKA α_1 was reduced by 30% in the presence of the EP_4 antagonist L-161 982 in hypertonic conditions for 24 and 48 hours, whereas the EP_1 antagonist SC 51089 had no effect.

Binding of PGE_2 to the EP_1 receptor is known to increase intracellular Ca^{2+} through the activation of PLC. In order to determine if the EP_1 antagonist SC 51089 was functional and specific to its receptor, the intracellular Ca^{2+} production was monitored with the fluorescent calcium indicator Fura-2AM with the Stallion Digital Hi-Speed Multi-Channel Imaging System. As illustrated in **Figure 10**, no change in intracellular Ca^{2+} was detected following PGE_2 or aldosterone stimulation, suggesting that the EP_1 pathway might not be functional in M-1 cells.

The effect of EP_4 antagonism by L-161 982 on NKA levels was investigated in more details. Normally, activation of the EP_4 receptor stimulates the activity of adenylate cyclase and leads to an increase in cAMP levels in the cell. Production of cAMP was detected by radioimmunoassay in control and high salt environments. As shown in **Figure 11**, a 25% increase in cAMP production was detected in the control environment following PGE_2 stimulation. This effect was most likely mediated by the EP_4 receptor since pretreatment with L-161 982 completely abolished the increase

in cAMP production. Interestingly, PGE_2 stimulation in a high NaCl environment did not increase cAMP, suggesting that the cAMP-dependent pathway might not be involved in the high salt-induced NKA protein expression. To support these observations, cAMP content was increased by stimulating the cells with 10µM forskolin in control and high salt environments. Levels of NKA α_1 were then assessed by immunoblotting. As shown in **Figure 12**, forskolin stimulation did not affect the levels of NKA after 24 hours but did increase cAMP by 50% in control salt.

A | 24 hours



B | 48 hours

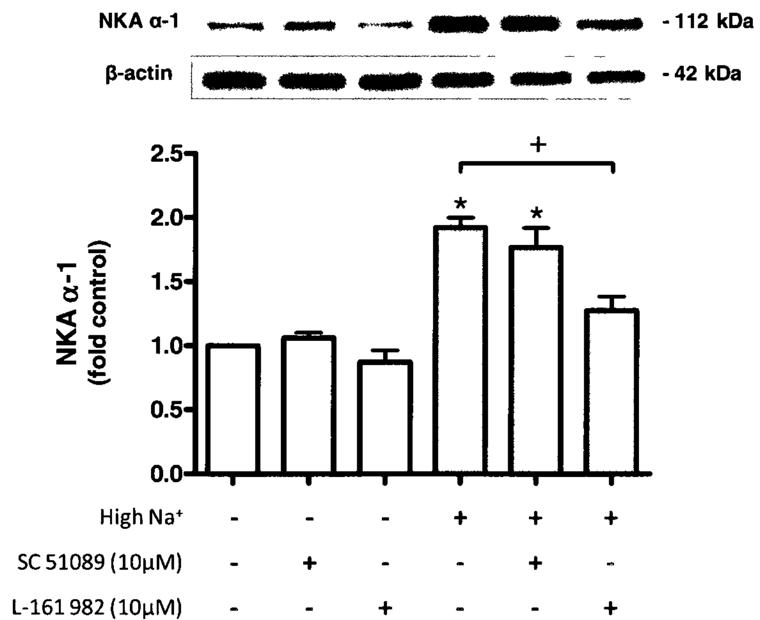


Figure 9 | Protein levels of NKA α_1 are reduced by L-161 982 but not SC 51089 in hypertonic conditions. M-1 cells were incubated in control (120mM NaCl) or high NaCl media (240 mM NaCl), in the presence of the EP_1 antagonist SC 51089 (10 μM) or the EP_4 antagonist L-161 982 (10μM). Total protein was analyzed by immunoblotting to examine the expression of NKA α_1-subunit after **A)** 24 hours (n=5, ANOVA, mean ± SEM * = $p < 0.001$, + = $p < 0.05$), and **B)** 48 hours (n=5, ANOVA, mean ± SEM + and * = $p < 0.001$). Expression of NKA α_1 was quantified by densitometry, adjusted for β-actin and expressed as fold control with ctrl=1.

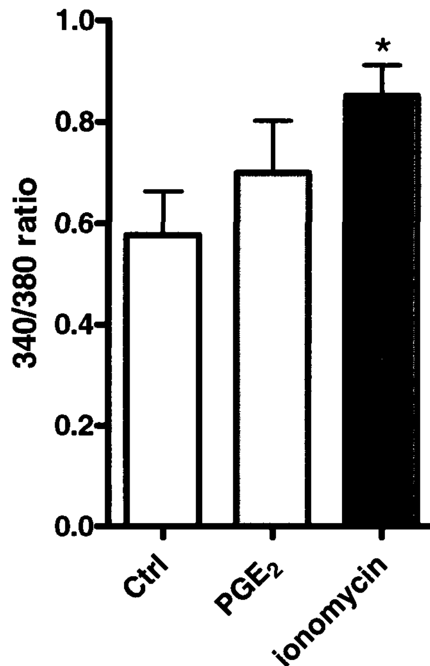


Figure 10 | PGE_2 stimulation does not increase intracellular Ca^{2+} production in control environment. M-1 cells were incubated in control media (120mM NaCl) for 24 hours. The coverslips were then mounted in a temperature-regulated chamber (37°C) continuously perfused (2mL/min) with Hanks' buffer. Cells were stimulated with PGE_2 (1µM) and the Ca^{2+} concentration was measured in multiple cells simultaneously by the Stallion Digital Hi-Speed Multi-Channel Imaging System, using an emission wavelength of 520 nm and alternating excitatory wavelengths of 343 nm and 380 nm. As a positive control, the cells were incubated in the presence of ionomycin (1µM). Results are expressed as 340/380 nm ratio of three region of interest randomly selected per stimulation. ANOVA, mean ± SEM (n= 3 to 4).

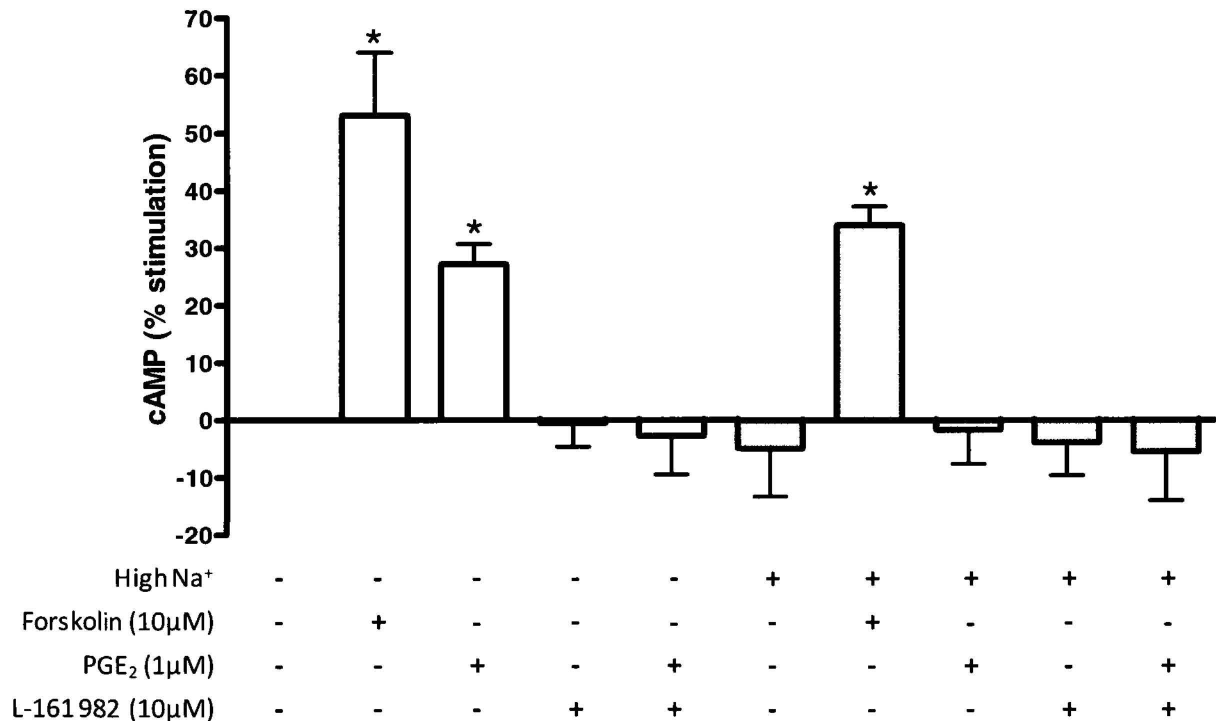


Figure 11 | PGE$_2$ alters cAMP production in control but not high salt environments. M-1 cells were incubated in control (120mM NaCl) or high NaCl media (240mM NaCl) for 24 hours. The cells were either pretreated with L-161 982 (10µM) before stimulation with PGE$_2$ (1µM) or untreated. The cAMP production was quantified by radioimmunoassay. As a positive control, cells were stimulated with forskolin (10µM). cAMP produced is expressed as % stimulation of control. ANOVA, mean ± SEM (n= 3). * = $p < 0.05$.

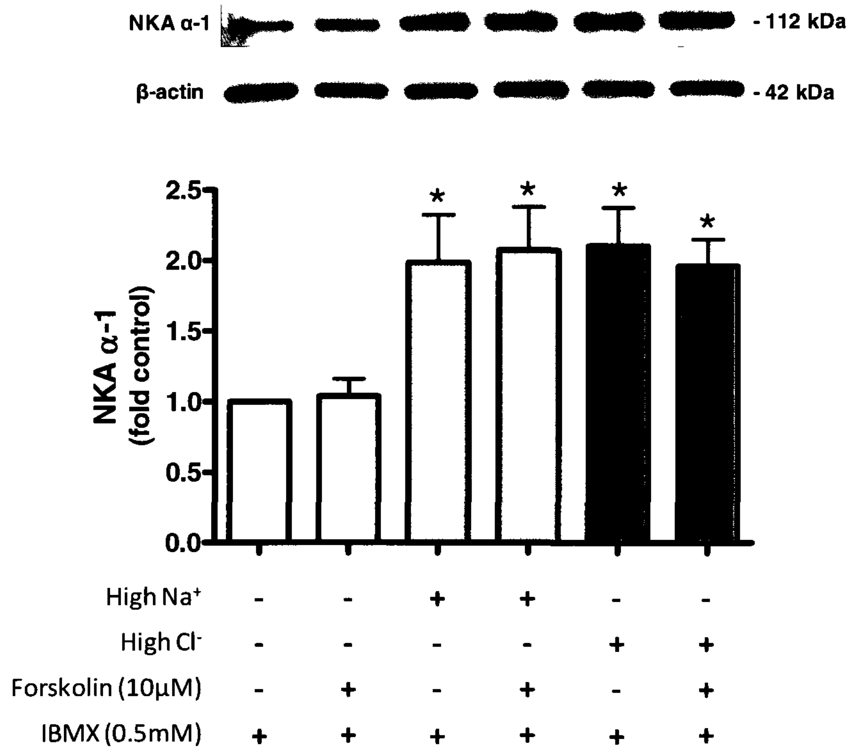


Figure 12 | Forskolin does not increase NKA α_1 protein levels in hypertonic conditions. M-1 cells were incubated in control (120mM NaCl) or hyperosmotic media (NaCl or choline chloride, 240mM) in the presence of forskolin 10μM and IBMX 0,5mM. Total protein was analyzed by immunoblotting to examine the expression of NKA α_1 subunit after 24 hours of treatment. Expression of NKA α_1 was quantified by densitometry, adjusted for β-actin and expressed as fold control with ctrl=1. n=4 ANOVA, mean ± SEM * = $p < 0.05$.

Interestingly, recent findings on EP_4 signaling have reported that EP_4 activation can stimulate a PI3K-dependent pathway (Fujino, Xu, Regan 2003). To determine if this pathway was activated by hypertonicity in M-1 cells, the cells were treated with the specific PI3K inhibitor LY294002 (50μM). A 30% reduction in the protein expression of NKA α_1 was observed after 24 hours of incubation in a hypertonic environment (**Figure 13**). Furthermore, this figure demonstrates that the inhibitory effects of the EP_4 antagonist L-161 982 and PI3K inhibitor LY294002 in high salt are not additive, suggesting that both components are acting on the same pathway. Activation of the PI3K pathway usually leads to the phosphorylation of Akt, the main downstream effector of this pathway. Akt phosphorylation was detected by immunoblotting, and a decrease in Akt phosphorylation was observed in our cells after 24 hours of incubation in high salt (**Figure 14**). This figure also illustrates that indomethacin (10μM) did not affect the ratio of phospho/total Akt in high salt, suggesting that the PI3K pathway is not activated in our cells.

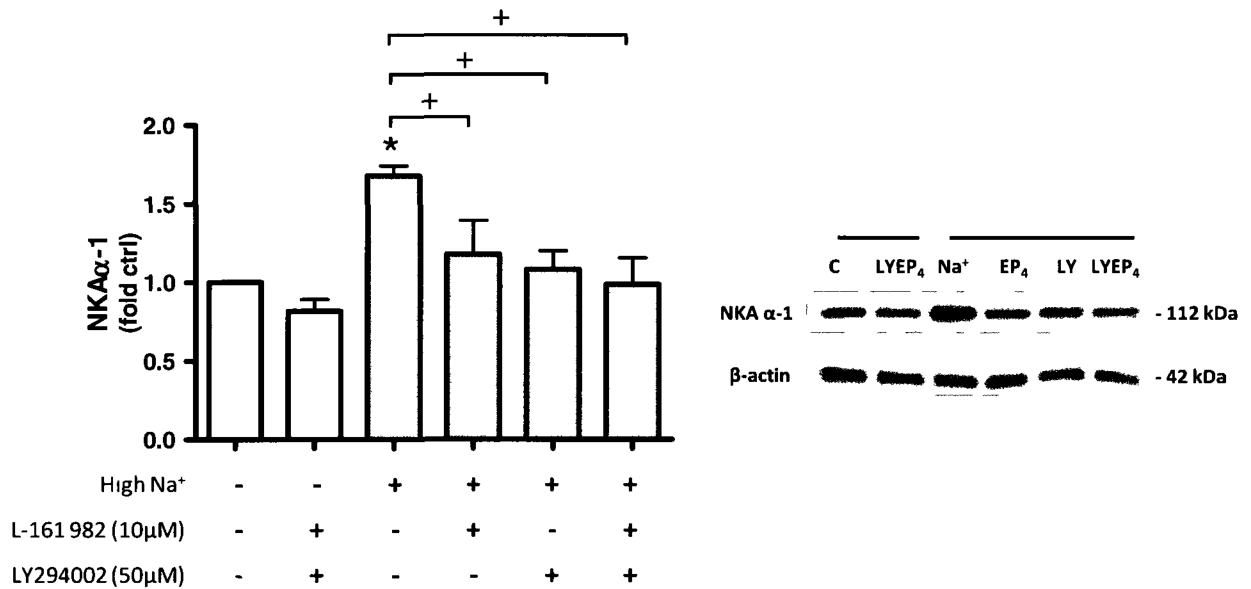
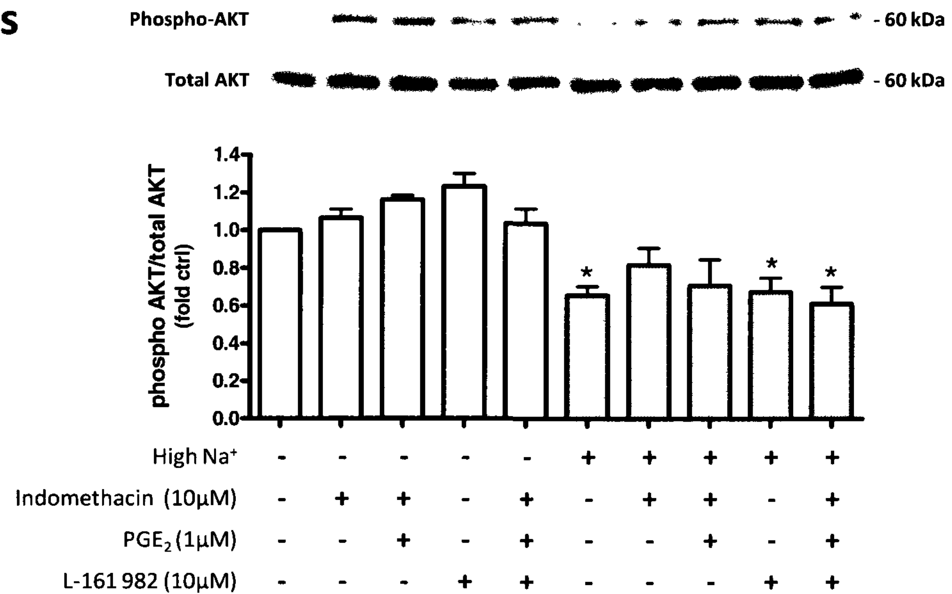


Figure 13 | LY294002 and L-161 982 attenuate high salt-induced NKA α_1 protein levels. M-1 cells were incubated for 24 hours in control (120mM NaCl) or high NaCl media (240 mM NaCl). The PI3K inhibitor LY294002 (50μM) and/or the EP_4 receptor antagonist L-161 982 (10μM) were added to the different media. Total proteins were analyzed by immunoblotting to examine the expression of NKA α_1 subunit after 24 hours of treatment. Expression of NKA α_1 was quantified by densitometry, adjusted for β-actin and expressed as fold control with ctrl=1. n=4, ANOVA, mean ± SEM * = $p < 0.01$, += $p < 0.05$



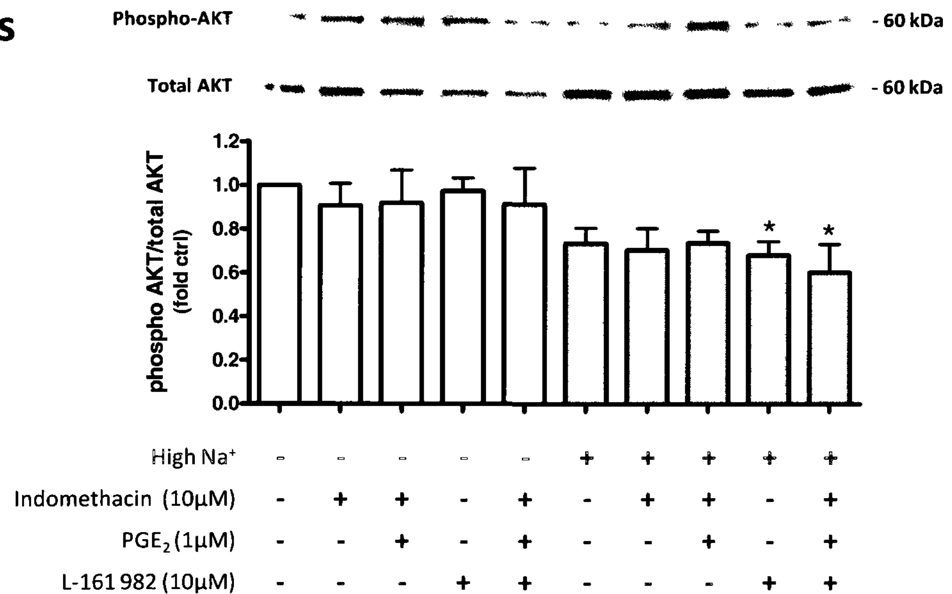


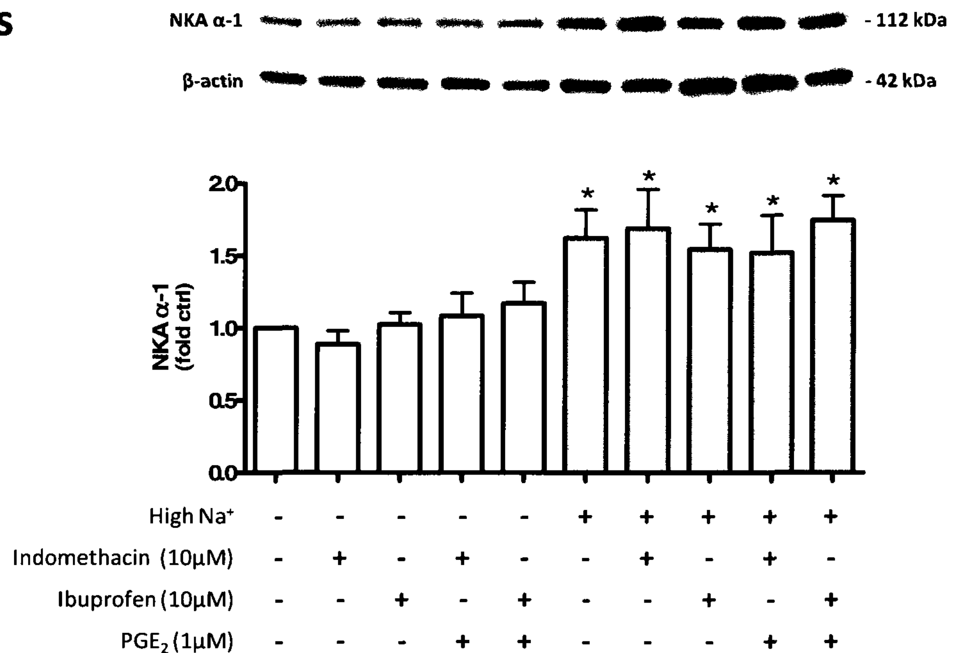
Figure 14 | Protein levels of phospho-Akt are reduced in hypertonic conditions. M-1 cells were incubated in control (120mM NaCl) or high NaCl media (240 mM NaCl), in the presence of indomethacin (10μM), PGE_2 (1μM) and the EP_4 antagonist L-161 982 (10μM). Total protein was analyzed by immunoblotting to examine the expression of phospho-Akt over total Akt after **A)** 24hours (n=4 to 8), and **B)** 48 hours (n=4 to 9) of treatment. Expression of Akt was quantified by densitometry and expressed as fold control with ctrl=1. ANOVA, mean ± SEM * = $p < 0.05$.

3.6 COX inhibition does not reduce NKA protein levels or activity in high salt.

The second experiment to assess if PGE_2 is involved in NKA regulation in high salt was to block the production of PGE_2 with COX inhibitors. The cells were incubated in hypertonic conditions in the presence of ibuprofen and indomethacin (10µM) for 24 and 48 hours. Levels of NKA were then detected by immunoblotting. As shown in **Figure 15**, no change was detected in the protein levels of NKA α_1 after 24 and 48 hours. Interestingly, there seemed to be a tendency of a decrease in NKA levels with ibuprofen treatment, as it numerically reduced from 2 to 1.5 fold. Furthermore, stimulation of the cells with PGE_2 (1µM) following a pre-treatment with indomethacin or ibuprofen (10µM) in high salt, did not affect NKA α_1 after 24 and 48 hours (**Figure 15**).

Estimation of NKA activity, by measuring the protein expression of NKA α_1 at the cell membrane, was also assessed in the same conditions. However, no change in NKA α_1 expression at the cell membrane was observed after 24 and 48 hours in the presence of indomethacin (10µM) and/or PGE_2 (1µM) in high salt (**Figure 16**). Finally, as illustrated in **Figure 7**, the salt-induced increase in PGE_2 was completely inhibited by pre-treatment with indomethacin and ibuprofen after 24 hours in high NaCl environment, confirming that NSAIDs are in fact reducing PGE_2 production.

A| 24 hours



B| 48 hours

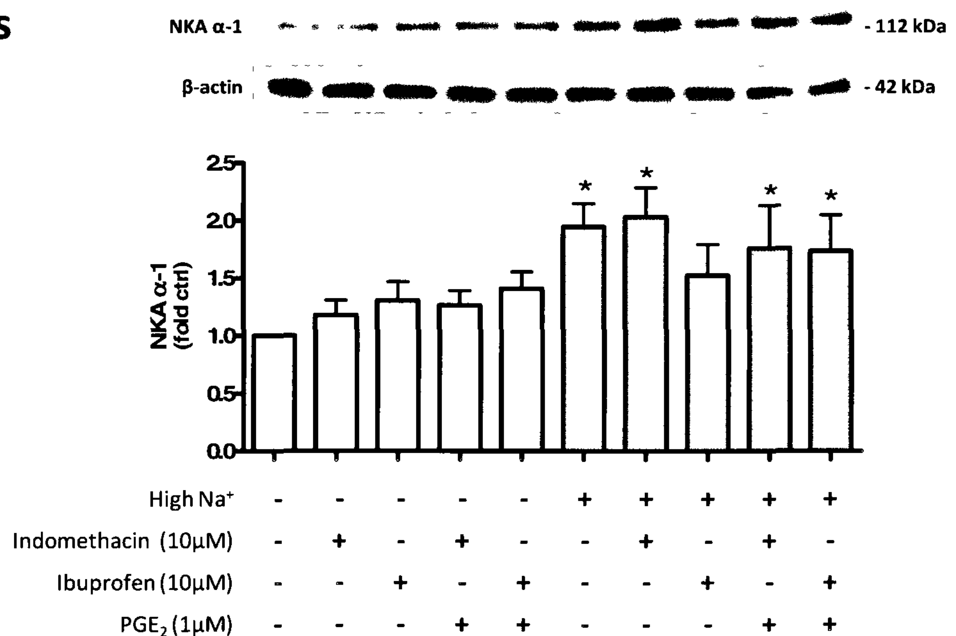
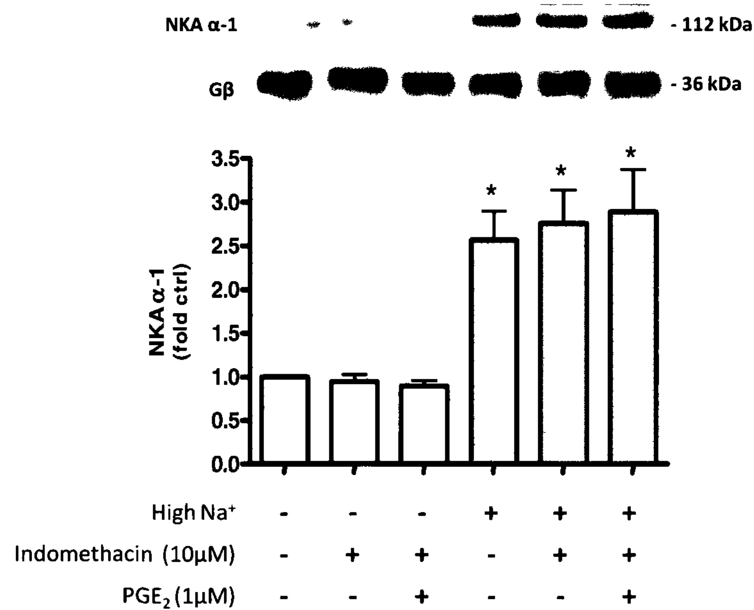


Figure 15 | Protein levels of NKA α_1 are not affected by indomethacin, ibuprofen and PGE_2 in hypertonic conditions. M-1 cells were incubated in control (120mM NaCl) or high NaCl media (240mM NaCl), in the presence of indomethacin (10μM), ibuprofen (10 μM) and PGE_2 (1μM). Total protein was analyzed by immunoblotting to examine the expression of NKA α_1 after **A)** 24hours (n=5), and **B)** 48 hours (n=5) of treatment. Expression of NKA α_1 was quantified by densitometry and expressed as fold control with ctrl=1. ANOVA, mean ± SEM * = $p < 0.05$.

A | 24 hours



B | 48 hours

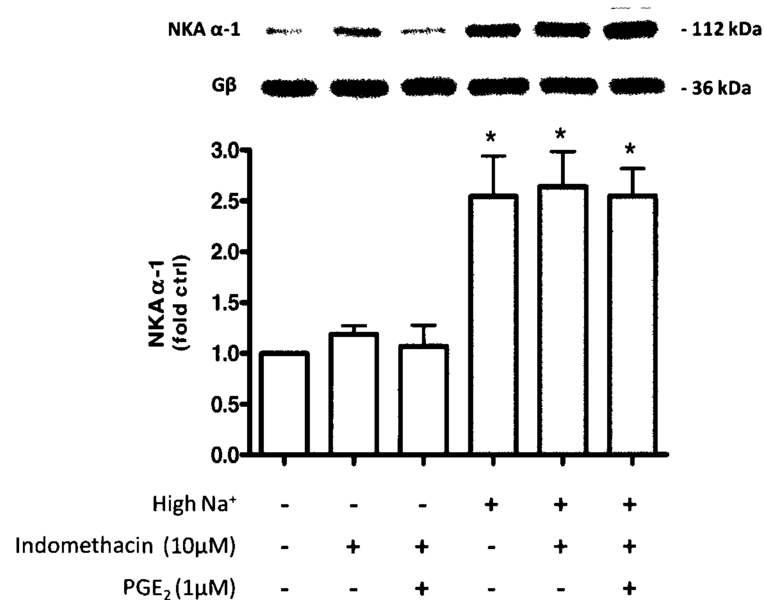


Figure 16 | Protein levels of NKA α_1 at the cell membrane are not affected by indomethacin and PGE_2 in hypertonic conditions. M-1 cells were incubated in control (120mM NaCl) or high NaCl media (240 mM NaCl) for 24 and 48 hours, in the presence of indomethacin (10µM) and PGE_2 (1µM). Cytosolic proteins were separated from cell membrane proteins by ultracentrifugation, and analyzed by immunoblotting to examine the expression of NKA α_1 subunit after **A)** 24 hours (n=6), and **B)** 48 hours (n=4). Expression of the cell membrane NKA α_1 was quantified by densitometry, adjusted for Gβ and expressed as fold control with ctrl=1. ANOVA, mean ± SEM * = $p < 0.001$

3.7 Hypertonicity and L-161 982 increase apoptosis.

Incubation in hypertonic conditions increased the number of dead cells in our experiments. Addition of the EP_4 antagonist L-161 982 (10µM) to the hypertonic media caused a further decrease in cell number. In order to investigate if this decrease in cell number is a result of a higher level of apoptosis, the protein levels of cleaved caspase-3 were quantified by immunoblotting. As illustrated in **Figure 17**, the levels of cleaved caspase-3 did not significantly increase in hypertonic environments after 24 and 48 hours. However, addition of L-161 982 (10µM) to this high salt environment increased the levels of cleaved caspase-3 by 2 fold after 24 hours and by 3 fold after 48 hours. In addition, the caspase-3 activity in the cell was analyzed by fluorescence. A 2 fold-increase in activity was observed in high salt, but addition of L-161 982 did not increase it further (**Figure 18A**). Treatment with indomethacin (10µM) did not affect caspase-3 activity after 24 hours in hypertonic conditions (**Figure 18B**). Finally, addition of PGE_2 (1µM) following treatment with L-161 982 (10µM) or indomethacin (10µM) did not reduce caspase-3 activity (**Figure 18A and B**).

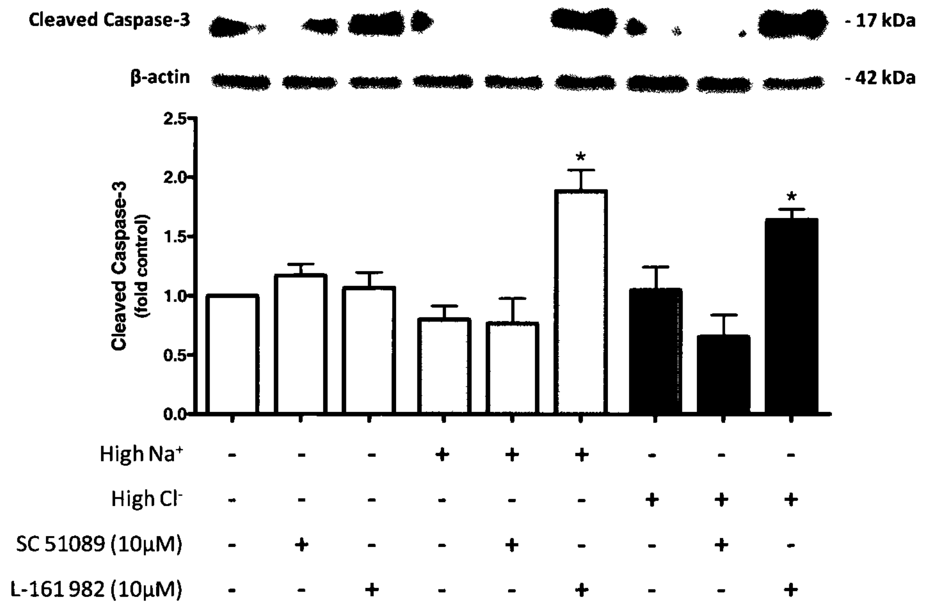
Induction of apoptosis usually increases the levels of the pro-apoptotic protein Bax and reduces the pro-survival protein Bcl-2. The ratio of Bax and Bcl-2 protein expression was measured by immunoblotting in our cells, but no change was detected in high salt or with the addition of L-161 982 (10µM) after 24 and 48 hours (**Figure 19**). To further investigate the apoptosis pathway, the protein levels of phospho-Akt were compared in control versus high salt, with and without L-161 982. Phosphorylation of Akt is reduced by 35% after 24 hours and by 20% after 48 hours

of incubation in the presence of a hypertonic environment (**Figure 14**). Treatment with L-161 982 (10µM) and indomethacin (10µM) had similar effects but treatment with PGE_2 (1µM) did not reverse the reduction in Akt phosphorylation. The protein levels of β-catenin, a downstream effector of Akt, were also analyzed by immunoblotting in hypertonic conditions. As shown in **Figure 20**, no significant change in levels were observed after 24 and 48 hours. However, there is a tendency for a decrease in β-catenin protein levels after 24 hours of incubation in high salt with a numerical reduction from 1 to 0.75. Treatment with indomethacin and PGE_2 did not affect β-catenin levels.

A decrease in cell number and an increase in apoptotic cells after 24 hours of incubation in high salt were demonstrated by Hoechst 33342 staining (**Figure 21**). **Figure 21** also illustrates a further increase in apoptotic cells following treatment with L-161 982 (10µM) in high salt. However, no change was observed with indomethacin (10µM) or PGE_2 (1µM) treatment in high salt.

Protein levels of phospho-p38 MAP kinase (MAPK) in hypertonic conditions were also assessed by immunoblotting. As illustrated in **Figure 22 A**, a 1.8-fold increase in phospho-p38 MAPK was observed after 24 hours of incubation in high NaCl. Treatment with L-161 982 (10µM) for 24 hours caused a 1.8 and 2.5-fold increase in phospho-p38 MAPK in control and high NaCl media respectively. Furthermore, a tendency for an increase in phospho-p38 MAPK was observed in the presence of indomethacin (10µM) and PGE_2 (1µM) in control and high salt conditions (**Figure 22B**). A numerical increase from 1 to 1.4 is seen, however, this increase did not reach statistical significance.

A| 24 hours



B| 48 hours

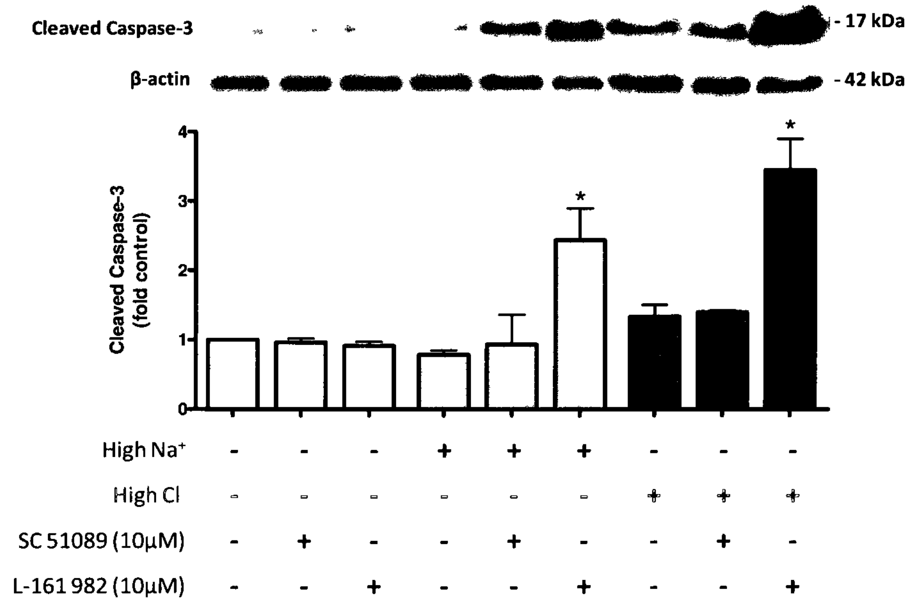
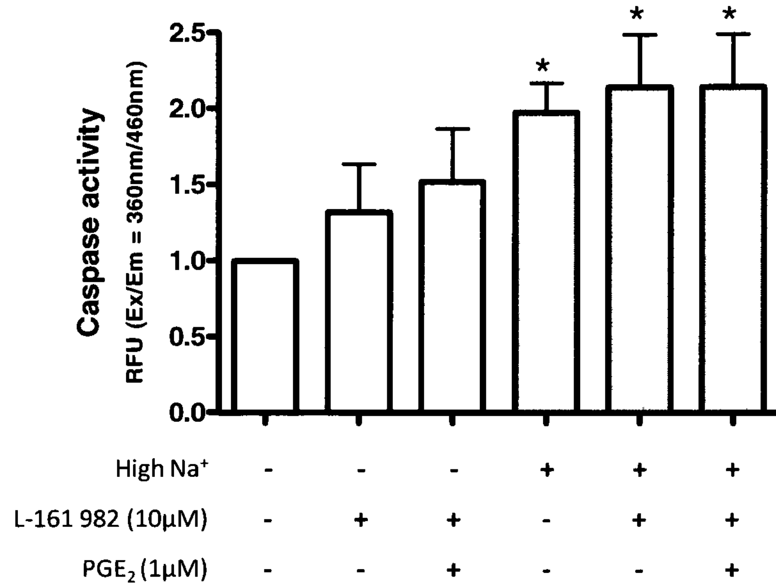


Figure 17 | L-161 982 increases the protein levels of cleaved caspase-3 in hypertonic conditions. M-1 cells were incubated in control (120mM NaCl) or hyperosmotic media (NaCl or choline chloride, 240 mM) in the presence of the EP_1 antagonist SC 51089 (10μM) or the EP_4 antagonist L-161 982 (10μM). Total protein was analyzed by immunoblotting to examine the expression of cleaved caspase-3 after **A)** 24 hours (n=4, ANOVA, mean ± SEM. * = $p < 0.01$), and **B)** 48 hours (n=3, ANOVA, mean ± SEM. * = $p < 0.05$) of treatment. Expression of cleaved caspase-3 was quantified by densitometry, adjusted for β-actin and expressed as fold control with ctrl=1.

A|



B|

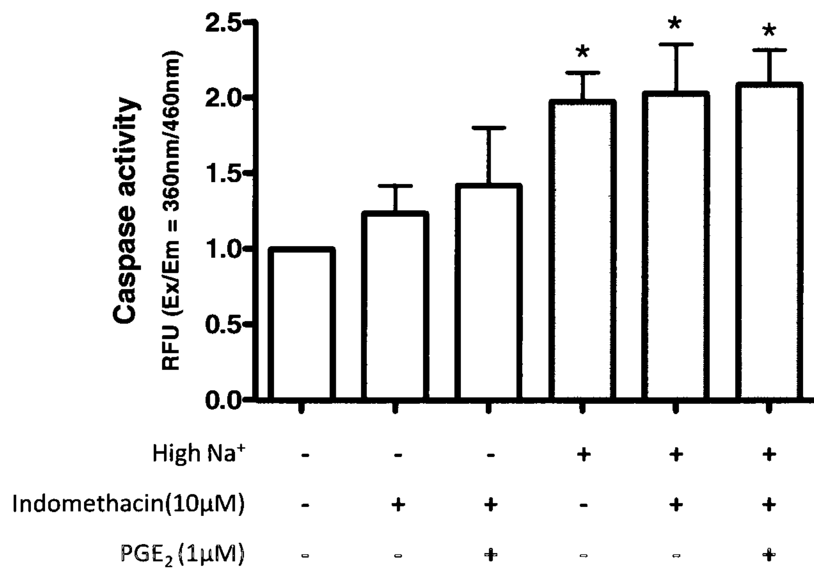
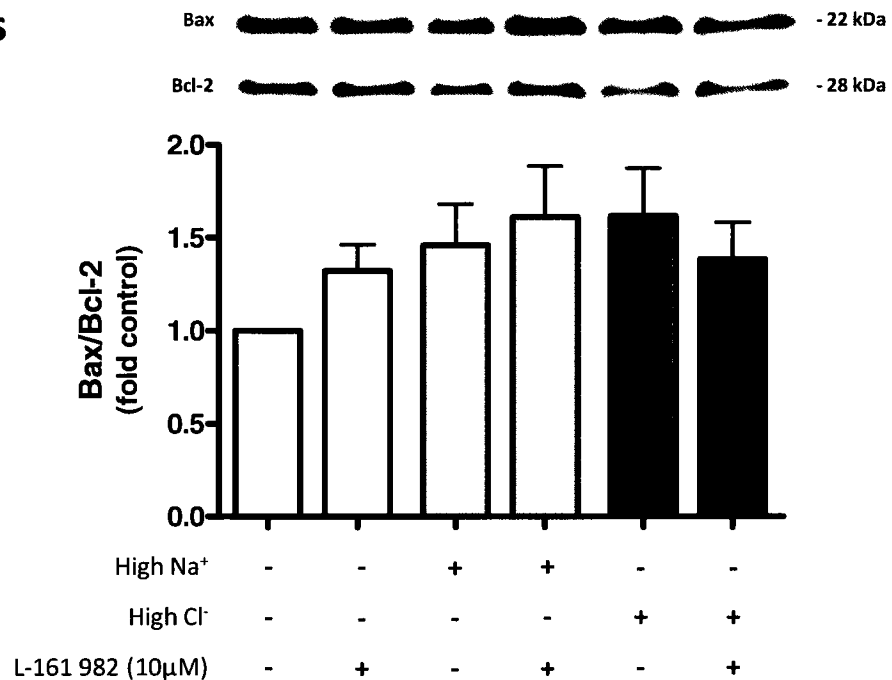


Figure 18 | Caspase activity is increased in hypertonic conditions. M-1 cells were incubated in control (120mM NaCl) or high NaCl media (240 mM NaCl) with the EP_4 antagonist L-161 982 (10μM), indomethacin (10μM), and PGE_2 (1μM) for 24 hours. Total protein was isolated and quantified to measure caspase activity in the presence of **A)** L-161 982 and PGE_2 (n=4, ANOVA, mean ± SEM *=$p < 0.01$), and **B)** indomethacin and PGE_2 (n=4, ANOVA, mean ± SEM *=$p < 0.05$). The capsase activity was determined by measuring the fluorescence emitted by the cleavage of the substrate Ac-DEVD-AMC (20 μM), minus the fluorescence emitted after pretreatment with the caspase inhibitor Ac-DEVD-CHO (400nM). The activity is expressed in relative fluorescence units (RFU=360/460nm).

A| 24 hours



B| 48 hours

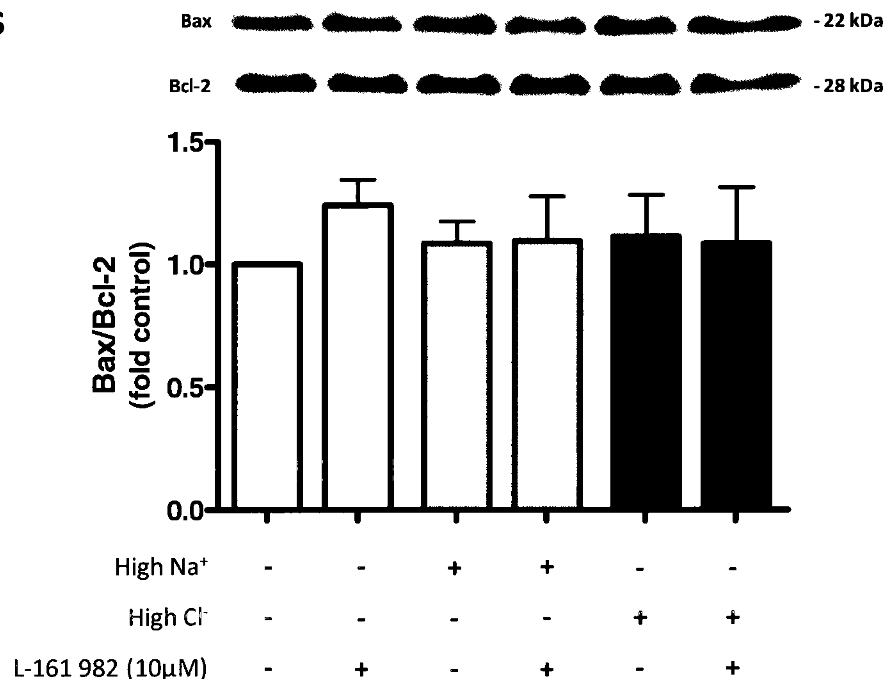
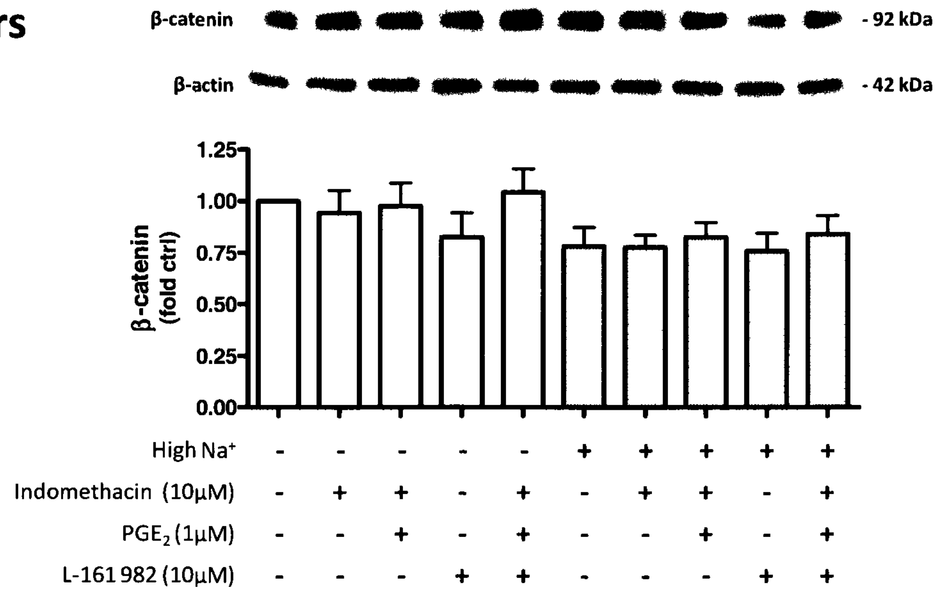


Figure 19 | Bax and Bcl-2 protein levels are not affected by L-161 982 and hypertonic conditions. M-1 cells were incubated in control (120mM NaCl) or hyperosmotic media (NaCl or choline chloride, 240mM) in the presence of the EP_4 antagonist L-161 982 (10μM). Total protein was analyzed by immunoblotting to examine the ratio of Bax and Bcl-2 after **A)** 24 hours (n=4), and **B)** 48 hours (n=5) of treatment. Expression of Bax and Bcl-2 was quantified by densitometry, adjusted for β-actin and expressed as fold control with ctrl=1.

A| 24 hours



B| 48 hours

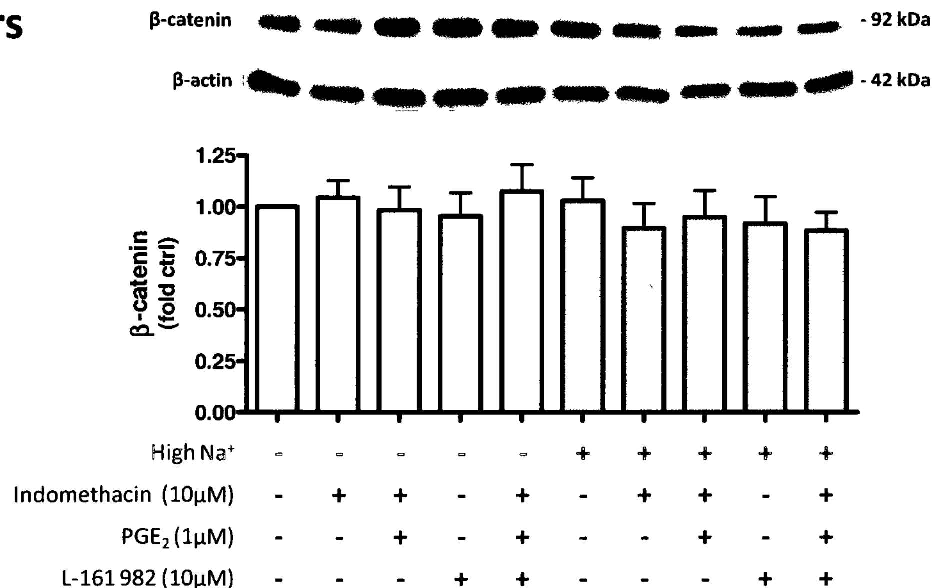
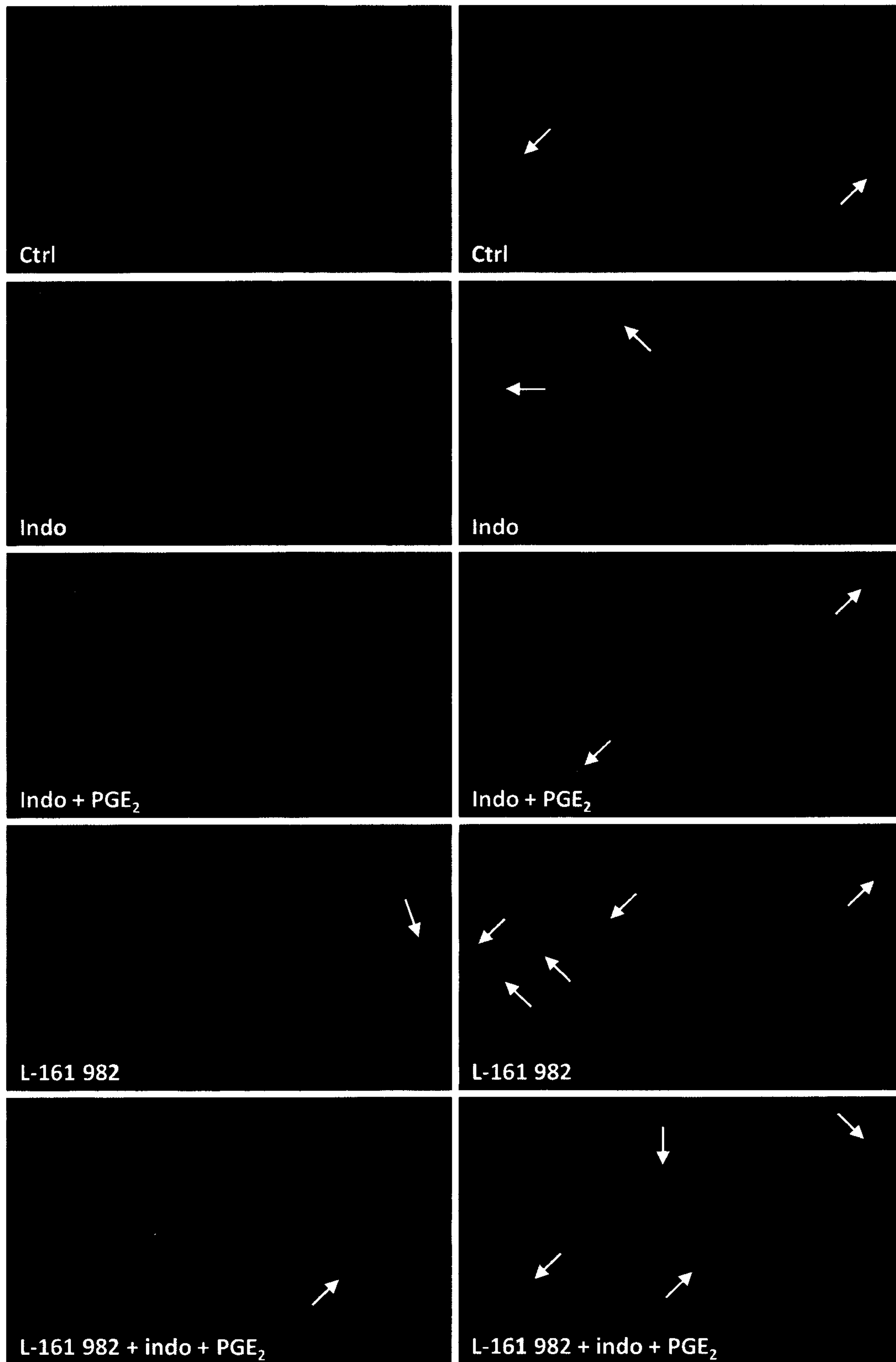


Figure 20 | Protein levels of β-catenin are not affected by hypertonic conditions. M-1 cells were incubated in control (120mM NaCl) or high NaCl media (240 mM NaCl), in the presence of indomethacin (10μM), PGE_2 (1μM) and the EP_4 antagonist L-161 982 (10μM). Total protein was analyzed by immunoblotting to examine the expression of β-catenin after **A)** 24hours (n=6), and **B)** 48 hours (n=5) of treatment. Expression of β-catenin was quantified by densitometry, adjusted for β-actin and expressed as fold control with ctrl=1.



Control medium
High NaCl medium
A|
Ctrl
Ctrl
Indo
Indo
Indo + PGE2
Indo + PGE2
L-161 982
L-161 982
L-161 982 + indo + PGE2
L-161 982 + indo + PGE2

B|

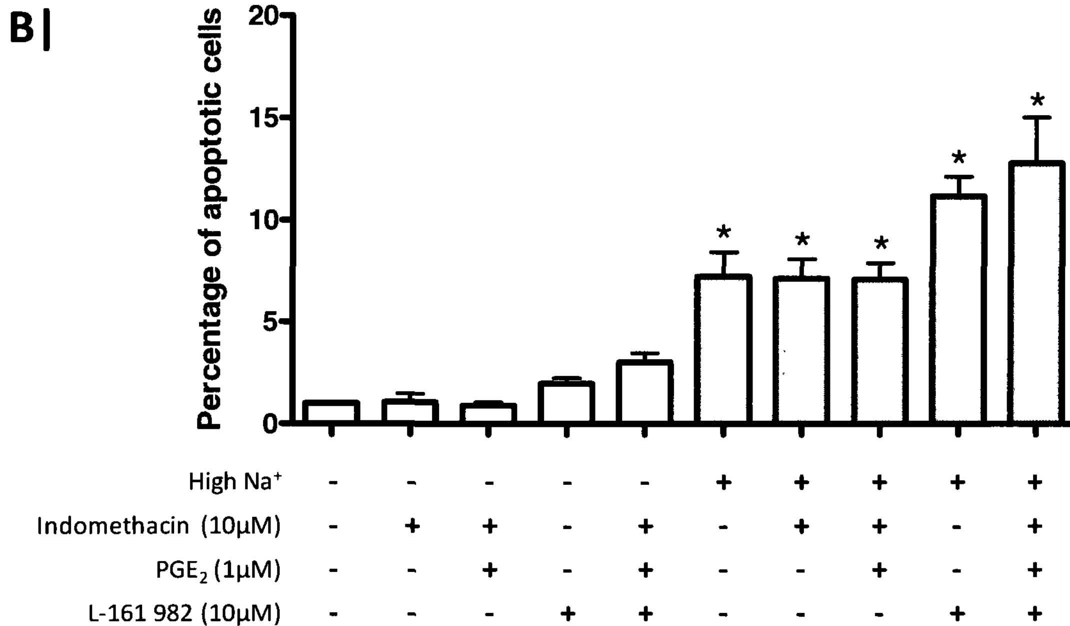
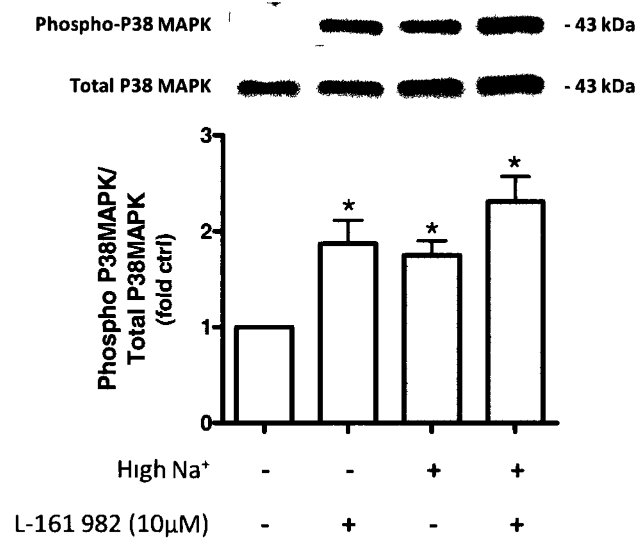


Figure 21 | High salt and L-161 982 treatment reduce cell number and increase apoptotis. M-1 cells were incubated in control (120mM NaCl) or high NaCl media (240mM NaCl), in the presence of indomethacin (indo, 10µM), PGE_2 (1µM) and the EP_4 antagonist L-161 982 (10µM) for 24 hours. The cells were stained with Hoechst 33342 and examined by fluorescence microscopy. **A)** Illustration of a representative field for each treatment. Cells with signs of apoptosis are indicated by the arrows. Magnification 400X. **B)** Apoptotic nuclei were counted in two random fields per coverslip, (two coverslip per treatments) and results are expressed as a percentage of total number of nuclei. n=4, ANOVA, mean ± SEM * = $p < 0.01$.

A|



B|

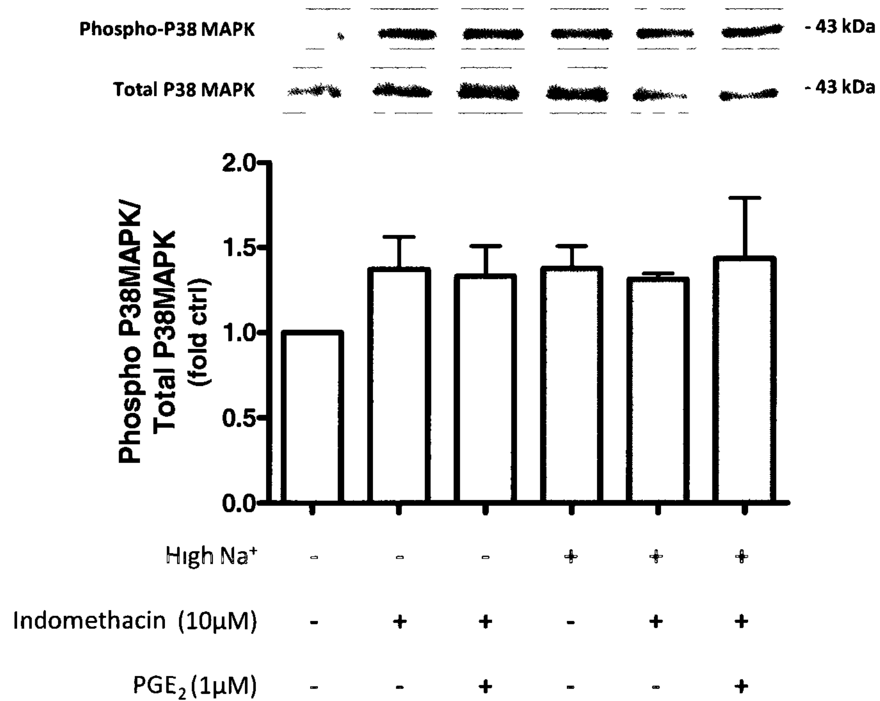


Figure 22 | Protein levels of phospho-p38 MAPK are increased by L-161 982 in hypertonic conditions. M-1 cells were incubated in control (120mM NaCl) or high NaCl media (240 mM NaCl), in the presence of indomethacin (10µM), PGE_2 (1µM) and the EP_4 antagonist L-161 982 (10µM). Total protein was analyzed by immunoblotting to examine the expression of phospho-p38 MAPK over total p38 MAPK after 24 hours in the presence of **A)** L-161 982 (n=4, ANOVA, mean ± SEM * = $p < 0.05$), and **B)** indomethacin and PGE_2 (n=4). Expression of p38 MAPK was quantified by densitometry and ratio was expressed as fold control with ctrl=1.

3.8 Hypertonicity and L-161 982 reduce DNA and protein synthesis.

Parallel to the increase in apoptosis, a reduction in DNA and protein synthesis was observed in hypertonic conditions. As shown in **Figures 23 and 24**, incorporation of ^{3}H-thymidine and ^{3}H-leucine was reduced by 45% and 40% respectively in a high NaCl environment. Addition of the EP_4 antagonist L-161 982 (10μM) to the high NaCl environment caused a further decrease in ^{3}H-thymidine and ^{3}H-leucine incorporation (55% and 50% respectively). Stimulation with indomethacin or PGE_2 did not affect the incorporation pattern.

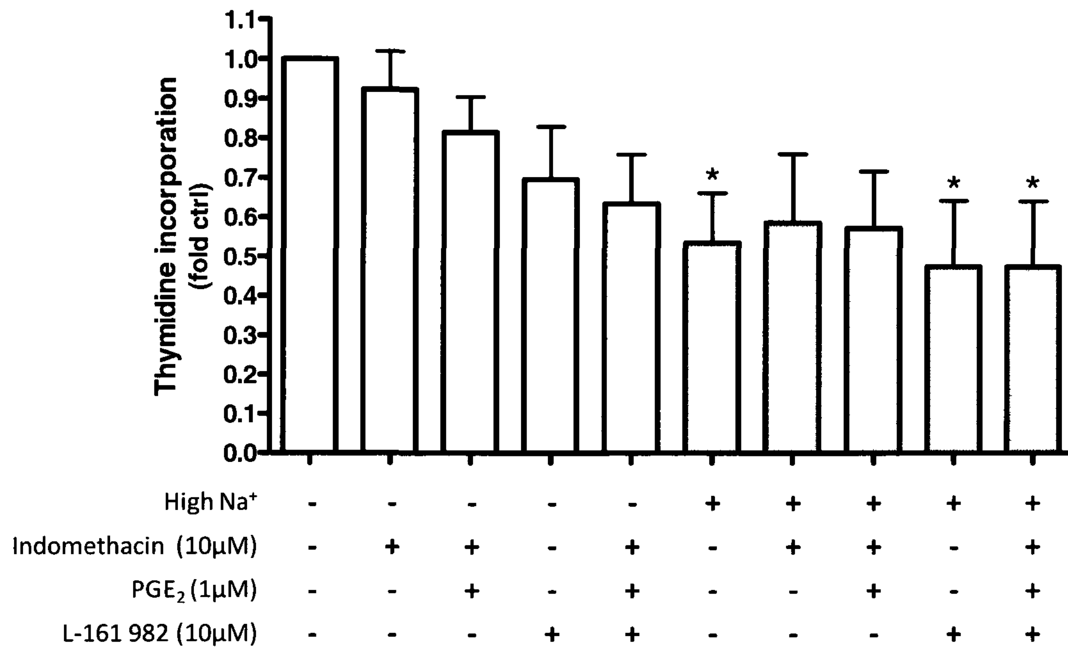


Figure 23 | DNA synthesis is decreased in hypertonic conditions. M-1 cells were incubated in control (120mM NaCl) or high NaCl media (240mM NaCl), in the presence of indomethacin (10μM), PGE_2 (1μM) and the EP_4 antagonist L-161 982 (10μM) for 24 hours. ^{3}H-thymidine was added to M-1 cells while they were being stimulated and thymidine incorporation was measured in disintegrations per minute and expressed as fold control of the mean. Each experiment was performed in triplicate. n=4, ANOVA, mean ± SEM * = $p < 0.05$

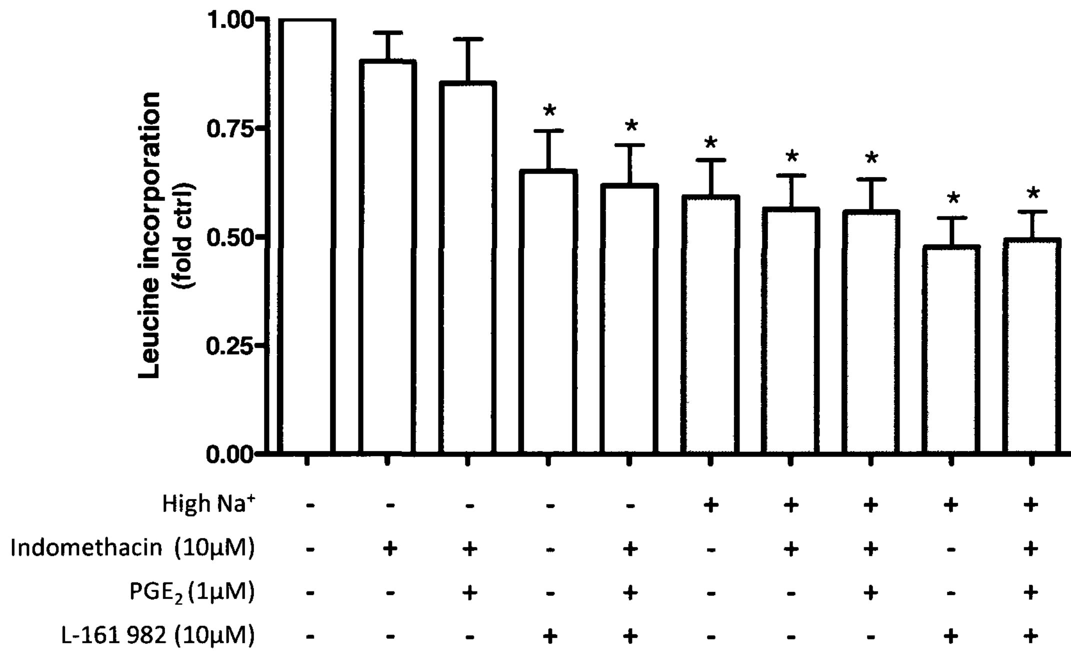
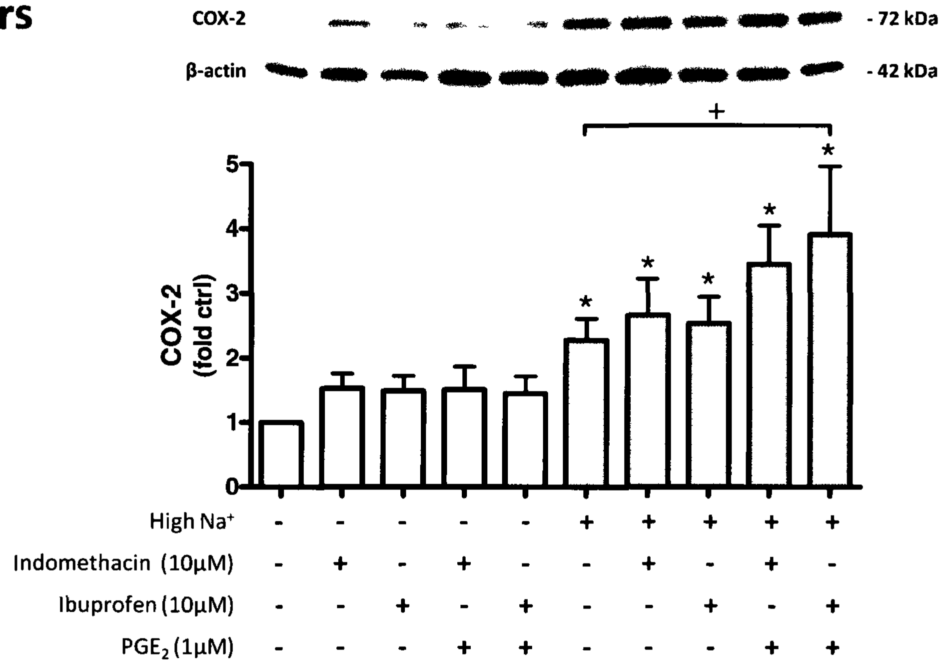


Figure 24 | Protein synthesis is decreased in hypertonic conditions. M-1 cells were incubated in control (120mM NaCl) or high NaCl media (240mM NaCl), in the presence of indomethacin (10μM), PGE_2 (1μM) and the EP_4 antagonist L-161 982 (10μM) for 24 hours. ^{3}H-leucine was added to M-1 cells while they were being stimulated and leucine incorporation was measured in disintegrations per minute and expressed as fold control of the mean. Each experiment was performed in triplicate. n=3, ANOVA, mean ± SEM * = $p < 0.05$.

3.9 COX-2 protein levels are increased by NSAIDs and PGE_2 in hypertonic conditions.

An interesting study by Steinert *et al.* (2009) described a positive feedback action of PGE_2 on COX-2 protein levels in high salt in renal medullary cells (Steinert *et al.* 2009). This feedback mechanism was investigated in our cell line, to determine if the high salt-induced PGE_2 production might be involved in the up-regulation of COX-2. The COX-2 steady state level of proteins was measured by immunoblotting in control and high NaCl environments. As illustrated in **Figure 25**, COX-2 levels were increased by 2.5 fold after 24 hours in high salt, and by 3 fold after 48 hours. Addition of indomethacin or ibuprofen (10μM) with or without PGE_2 (1μM) caused a further increase in COX-2 levels in control and high salt conditions. However, the increase observed after the addition of PGE_2 was not significantly different from the one observed with indomethacin or ibuprofen treatment alone.

A| 24 hours



B| 48 hours

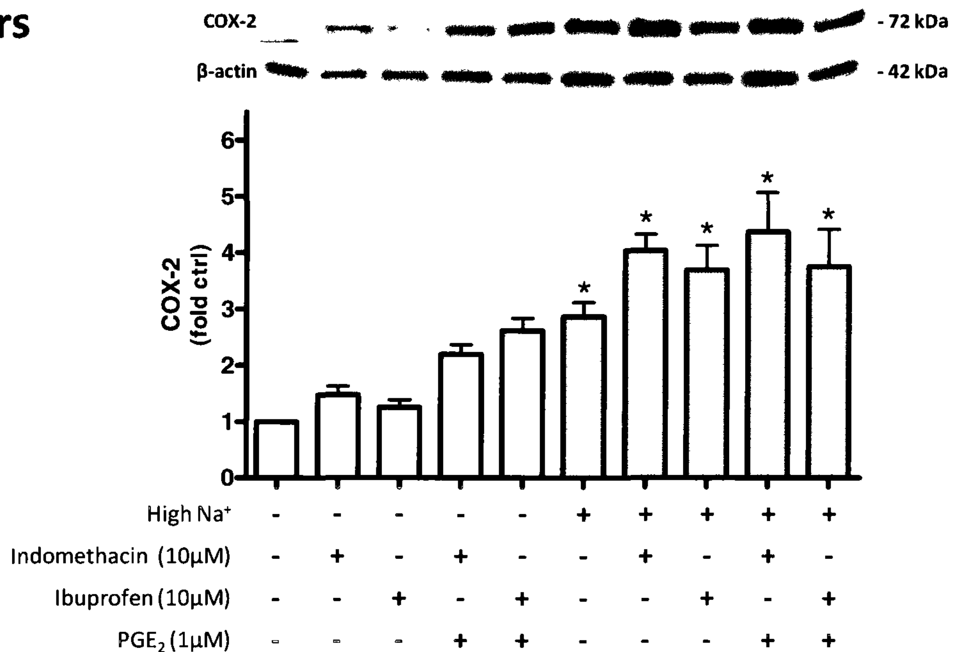


Figure 25 | Protein levels of COX-2 are increased in the presence of indomethacin, ibuprofen and PGE_2 in hypertonic conditions. M-1 cells were incubated in control (120mM NaCl) or high NaCl media (240mM NaCl), in the presence of indomethacin (10µM), ibuprofen (10µM) and PGE_2 (1µM). Total protein was analyzed by immunoblotting to examine the expression of COX-2 after **A)** 24hours (n=4) and **B)** 48 hours (n=5) of treatment. Expression of COX-2 was quantified by densitometry, adjusted for β-actin and expressed as fold control with ctrl=1. ANOVA, mean ± SEM * = $p < 0.05$.

3.10 NHE1 protein levels are not affected by hypertonicity.

This last experiment was performed to determine if other ion transporters of the CCD principal cells are affected by high salt-induced PGE_2 production. The protein levels of NHE1 were analyzed by immunoblotting. NHE1 is present at the basolateral membrane of principal cells, and it plays a major role in pH and cell volume regulation (Hill, Giesberts, White 2002). However, as shown in **Figure 26**, no change in NHE1 levels was detected after 24 hours of incubation in hypertonic conditions. Since no change was observed in NHE1 in high salt, we did not investigate NHE1 protein levels in response to PGE_2 or NSAIDs in our cells.

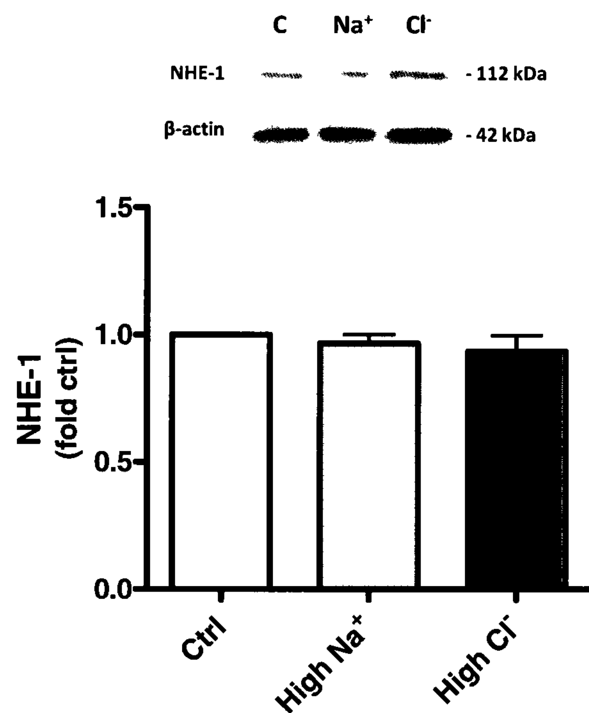


Figure 26 | Protein levels of NHE1 are not affected by hypertonic conditions. M-1 cells were incubated in control (120mM NaCl) or hypertonic media (NaCl or choline chloride, 240mM). Total protein was analyzed by immunoblotting to examine the expression of NHE1 after 24 hours (n=3) of treatment. Expression of NHE1 was quantified by densitometry, adjusted for β-actin and expressed as fold control with ctrl=1.

4. DISCUSSION

The first objective of this study was to determine if PGE_2 can regulate the protein levels and activity of NKA in hypertonic conditions in M-1 cells, and to determine which mechanisms are involved in this regulation. Our research is unique in that we investigated both the effects of high salt and PGE_2 on the regulation of NKA. Studies have demonstrated a potential action of PGE_2 or hypertonicity on NKA alone, but none have tried to link these two components in the CCD. Our results indicate an increase in NKA α_1 levels and activity in hypertonic conditions, as well as an increase in PGE_2 production after 24 hours. However, we could not demonstrate that PGE_2 regulates NKA in high salt. Inhibition of COX and antagonism of the EP receptors failed to reverse the high salt-induced NKA expression.

4.1 NKA and hypertonicity

First of all, our results indicate a 2 fold-increase in the steady state level of proteins of NKA α_1-subunit in M-1 CCD cells in hypertonic conditions (high NaCl, high choline chloride, high mannitol, 480 mOsm/kgH_2O), but not in the presence of high urea (480 mOsm/kgH_2O), which is an isotonic hyperosmotic environment. There is an important difference to make between hyperosmolality and hypertonicity. A hyperosmotic environment can be hypertonic or isotonic. In a hypertonic situation, the solute (eg. Na^+) cannot freely cross the cell membrane. Consequently, water leaves the cell by osmosis, resulting in a loss in cell volume. In an isotonic situation, the solute (eg. urea) can cross the cell membrane and cell volume is not changed.

Our results suggest that NKA is affected by hypertonicity rather than hyperosmolality in the CCD. Similar observations were made by Ohtaka *et al.* (1996) who reported an increase in NKA mRNA and activity in IMCD primary cell cultures in hypertonic conditions (Ohtaka *et al.* 1996). Conversely, a study by Vinciguerra *et al.* (2004) reported no change in NKA α_1 cell surface protein expression in hypertonic conditions in $mpkCCD_{cl4}$ cells, a mouse CCD cell line (Vinciguerra *et al.* 2004). Instead, they observed an increase in NKA α_1 cell surface protein expression in hypotonic environments, and suggested that it was caused by an increased apical Na^+ entry via ENaC. Further investigation in our cell line revealed an increase in NKA α_1 cell surface protein expression in hypertonic conditions, which contradict their findings. However, some differences exist between the two cell lines (M-1 and $mpkCCD_{c14}$) that may account for the discrepancy in the results. Both cell lines were derived by targeted oncogenesis with the SV40 large T-antigen, and are exhibiting a tight epithelium, a high transepithelial resistance and are expressing ENaC channels on their apical membrane. However, the protein levels of ENaC in $mpkCCD_{c14}$ cells seem to be more sensitive to aldosterone compared to M-1 cells (5). Differences in ENaC regulation between the cell lines might have important implications on the intracellular ion composition of the cell under specific conditions. For instance, hypertonicity might increase the intracellular Na^+ concentration in our cell line to a greater extends, leading to the activation of NKA. However, further investigation is needed to determine if NKA regulation is dependent of ENaC levels, and to determine if the regulation of ENaC by hypertonicity differs between the two cell lines. Studies in primary cell cultures should also be performed, since these

cells are more representative of the in vivo state of the collecting duct and they usually produce more physiological data.

The protein levels of NKA β_1-subunit were also analyzed, but no change was detected after 24 or 48 hours in hypertonic conditions. It is possible that the expression of the β-subunit is already high in our cells, making the synthesis of the α_1-subunit the limiting step in the assembly of the pump. Although work in this area is limited, studies performed on rat outer medullary (OMCD) and MDCK (distal epithelial) cells support this hypothesis (Buffin-Meyer *et al.* 1998; Tokhtaeva, Sachs, Vagin 2009). Both studies reported a higher level of β-subunit mRNA in the cells, suggesting that α-subunit is the rate-limiting step. However, this regulation seems to be tissue specific since the α-subunit is higher than β in alveolar epithelial cells (Yoshimura *et al.* 2008) and skeletal muscles (Taormino and Fambrough 1990) under basal conditions. Further characterization is needed to compare the relative expression of the different NKA subunits in the CCD under normal and hypertonic conditions.

It is well known that exposure to hypertonic environments can be harsh for the cells and can induce apoptosis. Studies have reported perturbations in Na^+ and K^+ homeostasis during apoptosis, and NKA is thought to play a role in this modulation (Bortner, Hughes, Cidlowski 1997; Xiao *et al.* 2002). In order to determine if the increase in NKA in high salt is a consequence of apoptosis, we treated our cells with anisomycin, a potent protein synthesis inhibitor. Interestingly, induction of apoptosis in our cells reduced the protein levels of NKA. Therefore, induction of NKA in high salt is probably a survival mechanism to resist the harsh

environment. In fact, exposure to hypertonicity results in cell shrinkage due to efflux of water. To compensate for this loss, an initial increase in Na^+ reabsorption occurs, perturbing the intracellular K^+ concentration (Bortner, Hughes, Cidlowski 1997; Xiao *et al.* 2002). To re-establish homeostasis, the cell needs to replace the intracellular Na^+ with K^+ and this can be done by activating NKA. Recent evidence suggested that depletion of intracellular K^+ is an important pro-apoptotic factor (Hughes *et al.* 1997; Hughes and Cidlowski 1999; Xiao *et al.* 2002; Yu 2003b). A low intracellular K^+ concentration has been shown to induce many important apoptotic processes, including, caspase-3 cleavage, cytochrome c release, and endonuclease activation (Hughes *et al.* 1997; Hughes and Cidlowski 1999; Yu 2003a). Subsequently, a decrease in NKA activity promotes apoptosis, by decreasing the intracellular K^+ concentration. Therefore, the high salt-induced NKA observed in our experiments is not a consequence of apoptosis, but it might be a mechanism to promote survival of the cell. However, further characterization is needed to determine the mechanisms promoting NKA protein expression.

4.2 PGE_2/EP receptor system and hypertonicity

Prostaglandins are highly produced in the kidney, and the collecting duct is one of the major sites of PGE_2 production (Bonvalet, Pradelles, Farman 1987). In the M-1 cortical collecting duct cells, COX-2 is the principal contributor of PGE_2 production (Nasrallah *et al.* 2001). Studies have indicated an increase in COX-2 protein levels in hypertonic conditions in the collecting duct, suggesting an increase in PGE_2 production (Lim *et al.* 2007; Yang *et al.* 1998). Our lab previously confirmed a 2 fold-increase in COX-2 protein levels, but not COX-1 in M-1 cells induced by

hypertonic conditions *(unpublished data, honour's project, 2008).* We now demonstrated a 3.5 fold-increase in PGE_2 production in high NaCl conditions by enzyme immunoassay. These results suggest that the high salt-induced PGE_2 is mainly produced by the COX-2 isoform.

To fully understand the PGE_2 signaling pathway, it is important to appreciate the fact that it can be modulated at multiple levels. In addition to the prostaglandin production level, the PGE_2 pathway also depends on the expression of the different EP receptors by the cell. Our lab previously demonstrated the presence of the EP_1, EP_3 and EP_4 receptors in M-1 cells by RT-PCR (Nasrallah *et al.* 2001). For the present study, we confirmed the presence of these three receptors by real-time PCR and we assessed their expression in hypertonic conditions, which has not been characterized yet. An important increase in EP_3 receptor mRNA expression (9 fold) and no change in EP_1 or EP_4 mRNA expression in hypertonic conditions were detected. Little is known about the modulation of CCD EP receptors in hypertonicity and their analysis is complicated by the lack of good antibodies. Only a few studies investigated the EP receptor expression in hypertonic conditions, but in other segments of the nephron. Interestingly, all these studies have reported an increase in EP_3 mRNA expression, which supports our findings. A first study by Jensen *et al.* (1999) characterized the EP receptor mRNA expression in rats fed with a normal or a high salt diet (Jensen *et al.* 1999). They observed an increase in EP_3 mRNA in the outer medulla following a high salt intake, and an increase in EP_4 mRNA in the glomerulus with a low salt diet. No change in EP_1 mRNA expression was observed in glomerulus or outer medulla regardless of salt intake. Another study by this group

indicated no change in EP_2 mRNA expression by dietary salt intake in the rat kidney medulla (Jensen *et al.* 2001). Finally, a study by Kim *et al.* (2009) demonstrated a decrease in EP_3 and EP_4 mRNA in rat inner and outer medulla following intake of the loop diuretic furosemide. However, they should have measured the expression of the receptors in high salt conditions in order to conclude that hypertonicity promotes EP_3 and EP_4 expression. Their study also indicated an increase in EP_3 and EP_4 mRNA expression in high salt in HepG2 cells, a human liver carcinoma cell line. They also demonstrated an increase in EP_4 mRNA and protein expression in MDCK cells following hypertonic treatment. Altogether, these studies indicate differences in the regulation of each EP receptors by hypertonicity according to their localization along the nephron. Each cell type in the kidney expresses different patterns of EP receptors in order to accomplish specific transport and biochemical functions. Taking into consideration that other prostaglandin pathways with similar or distinct functions are also present in these cells (IP, TP, DP, FP), it is not surprising to observe an interaction and crosstalk between these different pathways. These interactions might be critical for the cell to compensate for the changes in one of the EP pathways, by up-regulating or down-regulating other receptor pathways. Therefore, the increase in EP_3 receptor transcripts might be a consequence of the lack of activation of the inhibitory cAMP pathway caused by the diminished EP_4-cAMP response of M-1 cells in high salt. However, the presence of multiple prostaglandin signaling pathways specific to each section of the nephron renders this system quite complex, and further investigation is needed to determine the role of EP_3 in this hypertonic modulation.

4.3 The different EP_4 signaling pathways and hypertonicity

To determine the implication of the PGE_2/EP receptor system in NKA up-regulation in high salt, we blocked the PGE_2 signaling pathway at two different levels; we antagonized the EP receptors and we inhibited PGE_2 production with COX inhibitors. Our results indicate a decrease in high salt-induced NKA α_1 protein levels in the presence of the EP_4 antagonist, but not with the EP_1 antagonist. Since we did not obtain a Ca^{2+} response to PGE_2 in our cells, we concluded that the EP_1 pathway is not active in these cells. This is consistent with the findings of Huang *et al.* (2005) who demonstrated that PGE_2 and AVP cannot activate the Ca^{2+} signaling pathway in M-1 cells (Huang *et al.* 2005). Consequently, our work focused on the EP_4 signaling pathway. However, further investigations of the EP_4 signaling pathway made us conclude that the EP_4 receptor is probably not involved in NKA regulation. The effects observed with L-161 982 most likely result from apoptosis of the cells, caused by non-specific effects of the compound. Normally, binding of PGE_2 to the EP_4 receptor activates adenylate cyclase and increases cAMP production (Breyer *et al.* 1998). We were able to demonstrate an increase in cAMP following stimulation with PGE_2 in normal conditions. Since the EP_2 receptor is not present in our cells, this increase in cAMP is probably mediated by the EP_4 receptor. Moreover, pretreatment of the cells with L-161 982 completely prevented the production of cAMP in response to PGE_2, supporting this idea. We were expecting to detect high levels of cAMP in hypertonic environments since we demonstrated an elevated concentration of PGE_2 in these conditions. Surprisingly, the cAMP production in high salt was not increased. Since no change in NKA levels was

observed in control or hypertonic conditions, neither with PGE_2 or forskolin, we concluded that high salt-induced NKA protein expression is not mediated by an increase in cAMP.

These interesting findings led us to many possible explanations. First, we considered a possible down-regulation of the EP_4 receptor in hypertonic conditions in our cells. However, as mentioned earlier, the real-time PCR for the EP_4 receptor indicated a constant level of EP_4 mRNA in control and hypertonic environments. Although quantification of mRNA levels is a good indication of the abundance of a protein in the cell, it gives us little information about the post-transcriptional regulation of the protein. For example, studies have suggested a post-transcriptional regulation of the EP_4 receptor by PGE_2 itself. In fact, Nishigaki *et al.* (1996) demonstrated a rapid PGE_2 induced desensitization of the EP_4 receptor expressed in Chinese hamster ovary cells (Nishigaki, Negishi, Ichikawa 1996). The EP_4 receptor was also found to internalize rapidly in response to PGE_2 treatment in human embryonic kidney 293 cells (Desai and Ashby 2001). However, since there are no good antibodies to measure the protein levels of EP_4 receptor, we need to rely on signaling responses.

Recent findings suggest the presence of an alternative EP_4 signaling mechanism independent of cAMP production. There is evidence that the EP_4 receptor can activate a PI3K-dependent pathway, which would explain the low cAMP levels observed in hypertonic conditions (Fujino, Xu, Regan 2003). This signaling pathway was investigated in our cell line, and we observed a reduction in NKA α_1 protein levels in the presence of the potent PI3K inhibitor LY294002. In

addition, the effects of LY294002 and the EP_4 antagonist L-161 982 were not additive suggesting a common mechanism of action. However, in high salt conditions, or following PGE_2 stimulation, we could not detect any increase in the phosphorylation state of Akt, the main downstream effector of PI3K. Instead, we observed a decrease in Akt phosphorylation in hypertonic conditions, suggesting that PGE_2 may not be activating the PI3K pathway in our cells. However, since the PI3K pathway is a pro-survival pathway, it may be activated by other factors in hypertonic conditions, in order to prevent apoptosis. Inhibition of this pathway with LY294002 might have increased apoptosis levels, which in turn caused a reduction in NKA protein levels, as demonstrated with anisomycin treatment. Furthermore, it is possible that the EP_4 antagonist L-161 982 is not specific to the EP_4 receptor, but can also interfere with the PI3K pathway. This would explain why the inhibitory effects of L-161 982 and LY294002 are not additive. But further characterization is needed to confirm this hypothesis.

Finally, the most plausible explanation for the decrease in NKA protein levels caused by EP_4 antagonism is that L-161 982 acts on other components of the cell to increase cell death. In fact, we demonstrated that induction of apoptosis with anisomycin caused a decrease in NKA α_1 protein levels. In addition to our own work, many publications provide evidence that L-161,982 is a selective EP_4 antagonist (Aoudjit, Potapov, Takano 2006; Cherukuri *et al.* 2007; Cipollone *et al.* 2005). However, the pharmacological and toxicological properties of this product have not been fully investigated and it might be acting on other machinery in the cell,

such as DNA or protein synthesis. Therefore, it is hard to conclude from these results if PGE_2 is implicated in the regulation of NKA in high salt.

Another efficient way to determine the implication of PGE_2 in NKA regulation in high salt is to block the PGE_2/EP signaling pathway with COX inhibitors (indomethacin and ibuprofen). Blockade of PGE_2 production with COX inhibitors failed to reverse the high salt-induced NKA expression. The same observations were made for the cell surface protein expression of NKA. Inhibition of PGE_2 production by indomethacin and ibuprofen was confirmed by EIA. Furthermore, addition of PGE_2 to normal or hypertonic environments did not affect NKA α_1 protein levels. Our results are in opposition with previous studies performed in the CCD that reported an increase (Sakairi *et al.* 1995) or an inhibition in Na^+ reabsorption and NKA activity by PGE_2 (Cordova, Kokko, Marver 1989; Hébert, Jacobson, Breyer 1991; Satoh, Cohen, Katz 1992; Warden and Stokes 1993). However, these studies were performed in normal conditions, and they have not fully investigated the role of PGE_2 on NKA regulation in hypertonic conditions. Altogether, our findings suggest that the PGE_2/EP receptor system is not involved in NKA regulation in high salt. However, it is important to take in consideration the limitations of the M-1 cell line. In fact, our lab previously characterized the M-1 cell line and observed a lower number of principal cells (40%) compared to the initial number (75%) described by Stoos *et al.* (1991) (Nasrallah *et al.* 2001). A reduction in the number of principal cells can affect the sensitivity of the cells to PGE_2. It would be important to confirm these observations in a more representative cell line, or in primary cell culture, were

the percentage of principal and intercalated cells are more representative of the *in vivo* cortical collecting duct.

The low concentration in cAMP in hypertonic conditions despite the high PGE_2 content might be explained by the elevated expression of EP_3 receptors. This receptor is coupled to a G_i protein, which inhibits the adenylate cyclase and reduces cAMP production (Breyer *et al.* 1994). Our results indicate a 9 fold-increase in EP_3 mRNA in hypertonic conditions which might explain the low cAMP production in the presence of PGE_2. Further analysis of the EP_3 signaling pathway is required to determine if it can affect NKA expression in high salt. However, since inhibition of PGE_2 production by indomethacin and ibuprofen failed to reverse the high salt-induced NKA protein expression, the EP_3 receptor is probably not involved in this regulation. Furthermore, it was suggested that the EP_3 signaling pathway in the CCD was activated only in the presence of an AVP response (Hébert, Regnier, Peterson 1995). However, Huang *et al.* (2005) demonstrated that the AVP signaling pathway cannot be activated in M-1 cells (Huang *et al.* 2005). The lack of a functional AVP signaling pathway and the presence of multiple EP_3 splice variants in our cells complicate the investigation of this signaling pathway.

In summary, our study clearly indicates an increase in NKA α_1 protein levels and PGE_2 production in hypertonic conditions. We also demonstrated with EP receptor antagonism and COX inhibition that the high content in PGE_2 is not responsible for the increase in NKA protein levels. These two components are probably independent of each other, but the overall goal is the same; to maintain homeostasis. By increasing NKA expression to rapidly replace Na^+ ions for K^+, the

cell attempts to maintain a normal intracellular ion composition and prevents apoptosis in hypertonic conditions. The increase in NKA α_1 that we observed might be mediated by other factors such as an increase in intracellular Na^+. In fact, an interesting study by Vinciguerra *et al.* (2003) proposed the existence of an intracellular [Na^+]-sensing pathway that will activate a cAMP-independent protein kinase A to translocate NKA to the cell membrane (Vinciguerra *et al.* 2003). However, in their experiments, the collecting duct cells ($mpkCCD_{c14}$) were exposed to hypertonicity for a short period of time (30 minutes) and they did not detect any change in NKA protein levels. It would be interesting to determine if this pathway can increase NKA levels when the cells are exposed to a high salt environment for a longer period of time.

4.4 PGE_2 and other transporters of the CCD

Even if we demonstrated that PGE_2 has no effect on NKA protein levels in M-1 cells, PGE_2 has been described in many studies as an important regulator of salt and water transport along the nephron. However, the effects of PGE_2 on salt and water transport, and the consequences of the increased PGE_2 content in hypertonic conditions are not fully understood. PGE_2 might be acting on other transporters of the CCD, such as AQP2, ENaC or NHE1. In fact, our lab previously demonstrated the implication of PGE_2 in the inhibition of AVP-water transport via the EP_3 receptor (Hébert, Regnier, Peterson 1995). In hypertonic conditions, the cell might be stimulating PGE_2 production in an attempt to inhibit water reabsorption and prevent hypertension. Furthermore, studies have suggested a down-regulation of ENaC in

the medullary collecting duct in the presence of PGE_2 (Gonzalez *et al.* 2009). However the effects of hypertonicity on ENaC regulation by PGE_2 have not been characterized yet. In addition, PGE_2 was reported to stimulate NHE1 protein levels in MDCK cells (Rodriguez and Reyes 1995). Hypertonicity was also shown to affect NHE1 activity, in a tissue specific manner. In fact, studies have reported an inhibition of NHE1 in rat medullary thick ascending limb (Good 1995) while others have reported an activation of NHE1 in COS-7 cell and in CHO-K1 cells in hypertonic conditions (Garnovskaya *et al.* 2003). However, our results indicate no change in NHE1 protein levels in hypertonic conditions.

4.5 COX-2 feedback mechanism in hypertonicity

In an attempt to identify other possible targets of the high salt-induced PGE_2 content, protein levels of COX-2 were assessed. An increase in COX-2 protein levels in hypertonic conditions was observed after 24 and remained elevated after 48 hours. Interestingly, our results also indicate a further increase in COX-2 in the presence of indomethacin or ibuprofen with PGE_2, but also with indomethacin and ibuprofen alone. The increase in COX-2 levels by PGE_2 was also observed by Steinert *et al.* (2009), who described a positive feedback action of PGE_2 on COX-2 in high salt in renal medullary cells (Steinert *et al.* 2009). They suggested the implication of an EP_2-cAMP-PKA-dependent mechanism, independent of the EP_4 receptor. However, the EP_2 receptor is absent in the CCD and from M-1 cells (Nasrallah *et al.* 2001), and this mechanism does not explain the up-regulation in

COX-2 observed in the presence of indomethacin and ibuprofen alone. Interestingly, a study by Kundu *et al.* (2001) demonstrated an increase in COX-2 protein levels in the presence of indomethacin or NS-398, despite an inhibition in PGE_2 synthesis in a murine model of metastatic breast cancer (Kundu *et al.* 2001). Moreover, COX-2 protein expression was also increased in the rat small intestine and stomach following indomethacin administration, accompanied by a decrease in PGE_2 production (Takeuchi *et al.* 2004; Tanaka *et al.* 2005). In addition to these findings, COX inhibition was reported to increase the levels of 15-hydroprostaglandin dehydrogenase, which promotes PGE_2 metabolism, in MMDD1 cells (macula densa), in TT cells (human medullary thyroid carcinoma) and in HL60 cells (human promyelocytic leukemia cell) (Frenkian *et al.* 2001a; Frenkian *et al.* 2001b; Yao *et al.* 2008). The increased degradation of PGE_2 in the presence of COX inhibitors might explain the contradicting results that we obtained. In fact, the protein levels of COX-2 were measured after 24 hours of incubation in the presence of indomethacin or ibuprofen and PGE_2. The exogenous PGE_2 might have been all metabolized by 15-hydroprostaglandin dehydrogenase before exerting its effect on the cell. In that case, treatments with COX inhibitors alone or with PGE_2 are the same, which might explain the increase in COX-2 in both situations. The increased activity of 15-hydroprostaglandin dehydrogenase would also explain the low PGE_2 content of the cells in the presence of indomethacin and ibuprofen despite a high COX-2 protein expression. It would be interesting to assess the levels of 15-hydroprostaglandin dehydrogenase in our cells in the presence of indomethacin and ibuprofen.

4.6 Regulation of apoptosis by PGE_2 in hypertonic conditions

We observed a decrease in the number of cells in the presence of hypertonic media. Exposure to hypertonic conditions usually results in cell shrinkage, DNA breaks and eventually to cell death (Kultz and Chakravarty 2001; Michea *et al.* 2000). Our results indicate that this loss in cell number results from apoptosis. We demonstrated an increase in caspase-3 activity and phosphorylated p38 MAPK, a decrease in phosphorylated Akt and a higher number of apoptotic nuclei by Hoechst 33342 staining in hypertonic environments. We also observed a decrease in H^3-thymidine and H^3-leucine incorporation in high salt, suggesting an inhibition of the cell cycle. Interestingly, these effects were worsened by the addition of the EP_4 antagonist L-161 982. This last observation brought us to our second hypothesis; to determine if PGE_2 can promote cell survival via the EP_4 receptor in high salt. To support this idea, recent findings suggest that PGE_2 promotes cell survival of glomerular epithelial cells via the EP_4 receptor (Aoudjit, Potapov, Takano 2006). However, we demonstrated that both EP_4 signaling pathways (cAMP and PI3K) are not activated in high salt in M-1 cells. Moreover, our results indicate that inhibition of PGE_2 production with indomethacin, or addition of PGE_2 did not modulate the cell death response in high salt. Altogether, these results suggest that cell survival is not mediated by the PGE_2/EP receptor system. The increase in apoptosis observed in the presence of L-161 982 support our first conclusion, suggesting that L-161 982 probably interacts with other cellular signaling pathways, independent of EP_4 receptor activation.

4.7 Summary

This study investigated a potential role for PGE_2 in the regulation of NKA in hypertonic conditions, via activation of the EP_4 receptor. An increase in NKA α_1 and PGE_2 production was detected in the M-1 CCD cell line. Expression of EP_1, EP_3 and EP_4 receptors was also detected by real-time PCR in normal and hypertonic environments. The expression of EP_3 was increased in high salt while EP_1 and EP_4 expression remained constant. Preliminary studies with the specific EP_4 antagonist L-161 982, but not with the EP_1 antagonist SC 51089 reversed the high salt-induced NKA protein expression. However, blockade of the PGE_2 signaling pathway with COX inhibitors (indomethacin and ibuprofen) failed to reverse the elevated NKA protein levels in high salt, suggesting that PGE_2 is not involved in this modulation. Addition of PGE_2 to our control and high salt environments did not affect the levels of NKA, supporting the idea that PGE_2 is not involved in NKA regulation. Further characterization of the EP_4 signaling pathway suggested that the effect of L-161 982 on NKA expression probably results from an increase in apoptosis caused by non-specific effects of the compound. Activation of EP_4 by PGE_2 usually leads to an increase in cAMP via adenylate cyclase but this effect was totally abolished in hypertonic conditions. This effect might result from the up-regulation of the EP_3 receptor in high salt, but further characterization is needed. Fujino *et al.* (2003) demonstrated that EP_4 activation can trigger the PI3K pathway, independently of cAMP production (Fujino, Xu, Regan 2003). Inhibition of PI3K with the inhibitor LY294002 reduced the levels of NKA α_1 in high salt in our cells. However, the phosphorylation state of Akt, the main downstream effector of PI3K, was decreased

in high salt and in the presence of PGE_2, suggesting that the PI3K pathway is not activated by PGE_2 in our cells. However, further investigation is needed to determine if PI3K can phosphorylate other downstream effectors, without affecting Akt phosphorylation. An interesting target to evaluate would be the extracellular signal-regulated kinases (ERKs), since they were reported to be activated by PI3K in primary sensory neurons (Zhuang *et al.* 2004). Altogether, these results support the idea that L-161 982 induces apoptosis of the cells, causing a decrease in NKA α_1 protein levels. In fact, a further increase in cleaved caspase-3, phosphorylated p38 MAPK and apoptotic nuclei was observed in high salt in response to L-161 982.

These last observations contradicted our second hypothesis suggesting that PGE_2 promotes cell survival via EP_4 receptors. Our results indicate an increase in apoptosis in a hypertonic environment, but addition of PGE_2 or inhibition of COX had no effect on apoptosis levels. This suggests that PGE_2 may not be involved in CCD cell survival in hypertonic environments.

In conclusion, this study demonstrated the induction of NKA α_1 protein expression and PGE_2 in hypertonic environments and suggested that the up-regulation of PGE_2 is not involved in NKA modulation in high salt. However, these observations do not necessarily reflect the *in vivo* CCD, since the M-1 cell line does not possess functional EP_1-Ca^{2+} and AVP-cAMP signaling pathways. In the future, it would be interesting to investigate the possible mechanisms by which NKA is induced in high salt such as the mechanism proposed by Vinciguerra *et al.* (2003), who suggested an activation of NKA by a high intracellular Na^+ concentration (Vinciguerra *et al.* 2003). Furthermore, it would be interesting to identify possible

targets of the high PGE_2 content in hypertonic conditions. For instance, investigation of the EP_3 signaling pathway on AQP2 expression in hypertonicity would be a very interesting avenue.

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