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EFFECT AND SIGNALING PATHWAY OF EPINEPHRINE ON THE NA^+/K^+ ATPASE IN CACO-2 CELLS

by
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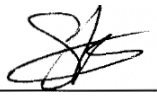

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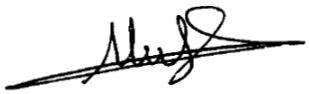

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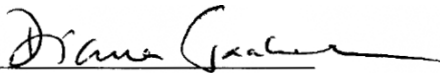

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AN ABSTRACT OF THE THESIS OF

Layla El Moussawi for Master of Science
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Title: Effect and signaling pathway of epinephrine on the Na+/K+ ATPase in Caco-2 cells

Epinephrine, a key stress hormone, has been shown to affect the physiological homeostasis of several biological processes in various body systems. In the gastrointestinal tract, stress has been associated with alterations in colonic functions leading to changes in water movements manifested as diarrhea or constipation. Colonic water movement is driven by the Na^+-gradient created by the Na^+/K^+-ATPase. Whether epinephrine acts via an effect on the Na^+/K^+-ATPase hasn't been studied before. In this work, we aim to investigate the effect of epinephrine on the Na^+/K^+-ATPase and to elucidate the signaling pathway involved by using CaCo-2 cells as a model. The activity of the Na^+/K^+-ATPase was assayed by measuring ATP hydrolysis in presence and absence of ouabain, a specific inhibitor of the enzyme. Epinephrine, added for 20 minutes, decreased the activity of the Na^+/K^+-ATPase by 50%. This effect was found to be mediated by α2 adrenergic receptors as it was fully abolished in the presence of Yohimbine an α2-blocker, but persisted in presence of other adrenergic antagonists. Furthermore, treatment with Rp-cAMP, a PKA inhibitor, mimicked epinephrine's negative effect and didn't result in any additional inhibition when both were added simultaneously. Treatment with indomethacin, PTIO, calphostin C, and PD98059, the respective inhibitors of PGE2, NO, PKC, and ERK completely abrogated the effect of epinephrine. In addition, an inhibitory effect, similar to that of epinephrine's, was observed upon incubation with PGE2, SNAP-1(NO generator), or PMA (PKC activator). PGE-2 was shown to act by binding to its EP1-receptors since its effect disappeared in presence of SC19220, an EP1-receptor antagonist. PGE2 failed to decrease the activity of the Na^+/K^+-ATPase in presence of PTIO and Calphostin C .Similarly, PMA's negative effect was not observed when added with PTIO, but persisted in the presence of indomethacin. Thus it can be concluded that epinephrine inhibits the Na^+/K^+-ATPase by the sequential activation of PGE2, PKC, and NO. Our findings reveal a negative regulatory role for epinephrine on the Na^+/K^+-ATPase in CaCo-2 cells that might underlie the stress –induced disruption in colonic water movement..

CONTENTS

ILLUSTRATIONS

ABBREVIATIONS

AC	Adenylyl cyclase
AMPK	5'adenosine monophosphate activated protein kinase
AP-1	Activator protein 1
AP-2	Activator protein 2
AR	Adrenergic receptor
ATP	Adenosine triphosphate
BAEC cells	Bovine aortic endothelial cells
Caco-2	Human colon adenocarcinoma cells
CAM	Cell adhesion molecule
cAMP	Cyclic adenosine monophosphate
cGMP	Cyclic guanosine monophosphate
CHO cells	Chinese hamster ovary cells
c-jun	Cytosolic jun
COS-7 cells	Monkey kidney fibroblast cells
Cox	Cyclooxygenase
CRH	Corticotrophin releasing hormone
C-terminal	Carboxy-terminal
DAG	Diacylglycerol
DARPP-32	Dopamine and cAMP regulated phosphoprotein
eNOS	Endothelial nitric oxide synthase
EP	E-prostanoid
EPAC	Exchange protein directly activated by cAMP
ERK	Extracellular signal regulating kinase
GDP	Guanosine diphosphate
GI	Gastrointestinal
Grb2	Growth factor receptor bound protein 2
GTP	Guanosine triphosphate
HEK-293 cells	Human embryonic kidney cell line
Hela cells	Immortalized cervical cancer cells
HepG2 cells	Human hepatoma cell line
HIF	Hypoxia inducible factor
HT-29 cells	Human colon adenocarcinoma cells
IBS	Irritable bowel syndrome
IL-10	Interlukin-10
IMCD cells	Inner medullary collecting duct cells
iNOS	Inducible nitric oxide synthase
IP3	Inositol triphosphate

KD	Kilo Dalton
LLCPK1	Kidney proximal tubule cell line
MAPK	Mitogen activated protein kinase
MAPKK	MAPK kinase
MAPKKK	MAPKK kinase
NF-KB	Nuclear factor kappa-light-chain-enhancer of activated protein B
nNOS	Neural nitric oxide synthase
NO	Nitric oxide
N-terminal	Amine-terminal
OK cells	Opossum Kidney cells
PC12 cells	Rat phaeochromocytoma
PDE2	Phosphodiesterase E2
PG	Prostaglandin
PGE2	Prostaglandin E2
PGG2	Prostaglandin G2
PI3K	Phosphoinostide -3 kinase
PIP2	Phosphatidyl inositol 4,5-bisphosphate
PKA	Protein Kinase A
PKB(AKt)	Protein Kinase B
PKC	Protein Kinase C
PKG	Protein Kinase G
PLA2	Phospholipase A2
PLC	Phospholipase C
PMA	Phorbol 12-myristate 13 acetate
PP5	Protein Phosphatase type 5
PTIO	2-(4-Carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide
PyK2 kinase	Phospho -Tyr881 kinase
Rac-1	Ras-related C3 botulinum toxin substrate 1
Raf	RAF proto-oncogene serine/threonine-protein kinase
Rap-1	Repressor activator protein -1
Ras	Rat sarcoma viral oncogene homologue
RpcAMP	Rp-Cyclic 3′,5′-hydrogen phosphorothioate adenosine triethylammonium
Ser	Serine residue
sGC	Soluble guanylyl cyclase
SH-sY5Y cells	Human neuroblastoma cells
SNAP-1	S-Nitroso-N-acetyl-DL-penicillamine
SNP	Sodium nitroprusside dihydrate Son of sevenless
SOS	Son of sevenless
STAT-1	Signal transducer and activator of transcription 1

Thr	Threonine residue
TNF	Tumor necrosis factor
Tyr (Y)	Tyrosine residue
VEGF	Vascullar endothelial growth factor

CHAPTER I

INTRODUCTION

Stress, whether physical or mental, is a ubiquitous condition that is part of our everyday life. When confronted with potential stressors, the brain triggers a cascade of physiological reactions, known as the ‘’fight or flight response’’, to ensure the individual’s survival and adaptation to the threatening events (McEwen, 2008). Neural inputs from the brain stimulate the hypothalamus to release CRH (corticotrophin releasing hormone) which, in turn, activates both the sympathetic-adrenal medulla and pituitary-adrenal cortex axes, resulting in the respective release of the primary stress hormones: epinephrine and cortisol into the blood stream(Kemeny, 2003).Together these hormones trigger the physiological deviations from homeostasis observed in the different systems of the body (cardiovascular, immune, endocrine, reproductive, respiratory,etc…) during the acute stress response (Chrousos& Tisgos,2002; Khansari D. *et al .,*1990; McEwen B.S. , 2008). A key target of the stress reaction appears to be the gastrointestinal tract (GI) whereby the prevalence and the severity of several GI disorders were found to correlate with anxiety , depression, and neuroticism (Bhatia & Tandon,2005). Among the various GI diseases, the role of stress in the pathophysiology of irritable bowel syndrome (IBS) has been extensively studied. IBS is considered one of the most prominent chronic gastrointestinal disorders, and is mainly characterized by abdominal pain and discomfort due to either frequent diarrhea or constipation (Everhart & Renault, 1991). These symptoms have been attributed to a number of factors including: imbalance of autonomic

nervous system (Mayer, 2000), changes in the activity of certain regions of the brain (Mayer, 2000), deregulated expression of pre- and postsynaptic receptors (Mayer, 2000), abnormal neuroendocrine secretion (Posserud *et al.*, 2004), alteration in colonic motor response and contractility (Narducci *et al.,* 1985),and certain visceral immune perturbations (Cremon *et al.,*2009;Spiller *et al.,*2012).

Epinephrine, a key stress hormone, was reported to affect water movement across the epithelium of certain tissues such as the human eye (Erickson-Lamy KA & Nathanson JA, 1992), lungs (Lane *et al.,* 1998), and kidneys (Hawk *et al.,* 1993) .Nonetheless, a potential role of epinephrine, in the alteration of colonic water movement and the development of IBS symptoms, or even their exacerbation, has not been investigated before.

Water movement across epithelial layers of the colon is governed by the Na+ gradient created by the Na^+/K^+-ATPase. By pumping 3Na+ions to the outside of the cell in exchange for $2K^+$ ions to the inside, the Na^+/K^+-ATPase establishes and maintains a low intracellular Na^+ concentration which drives Na^+ ions to flow down their electrochemical gradient from the lumen into the cytosol . This Na^+ diffusion generates osmotic forces that cause water molecules to follow across the plasma membrane (Sandle, 1998).

Consequently ,an alteration in the activity of the Na^+/K^+-ATPase was found to modify the direction and rate of net water transport as detected in the intestines of deoxycorticosterone acetate- injected mice (Charney *et al.,*1975),in the ileum of methylprednisolone -pretreated rats (Charney & Donowitz,1976), in rat proximal tubular cells following high Na^+-diet(Campo *et al.,*1990) ,and in rat brain during acute cerebral ischemia (Mintorovitch *et al.,*1994).

As an attempt to understand the relation between the stress reaction and colonic water movement, we aimed to study the effect of epinephrine on the activity of the Na^+/K^+-ATPase in colon adenocarcinoma cells (CaCo-2), and to elucidate its underlying mechanism of action.

CHAPTER II

LITERATURE REVIEW

A. Catecholamines

Catecholamines, as their name implies, are amines that possess a catechol (3, 4-dihydroxyphenyl) group (Nagastu, 2006).They are water soluble compounds that affect a wide range of tissues (Molinoff & Axelrod,1971) and act as physiological modulators towards homeostasis in response to the varying environmental perturbations (Arun,2004). Three distinct catecholamines were indentified *in vivo*: dopamine, noradrenaline (norepinephrine), and adrenaline (epinephrine) (Grace *et al.,* 1997). All are derived from L-tyrosine by a sequential cascade of reactions that will first produce dopamine from DOPA, followed by further metabolic modifications to give norepinephrine and epinephrine (Blaschko, 1939; Nagastu& Levitt, 1964).

Fluorescence histochemical analysis permitted the *in vivo* localization of catecholamines (Jonsson, 1971). While dopamine appeared to act strictly as a neurotransmitter in the central nervous system (Armstrong *et al.,* 1982), epinephrine and norepinephrine were shown to be released as both neurotransmitters from central and peripheral (sympathetic) neurons (Armstrong *et al.,* 1982), and as hormones from chromaffin cells of the adrenal medulla (Wood *et al.,* 1971; Silverberg *et al.,* 1978)

Whether they are secreted into a synaptic cleft or into the blood stream , epinephrine and norepinephrine exert their effects by binding to the cell surface adrenergic receptors (AR)

at their target site (Furchgott,1959).Structural and functional analysis allowed the subdivision of adrenoceptors into three α1 (α1A,α1B,α1D),three α2 (α2A,α2B,α2C) , and three β(β1,β2,β3) subtypes (Bylund*et al.,*1994) , resulting in a total of nine mammalian adrenoceptors identified so far. All adrenergic receptors however, are classified as members of the G-protein coupled receptors family characterized by seven-transmembrane domains , with an extracellular N-terminus and a cytosolic C-terminus, and interact through their cytoplasmic loops with a specific type of G-proteins (Kobilka,1992).

1. Catecholamines and the Na^+/K^+-ATPase

The possibility of interaction between catecholamines and the Na^+/K^+-ATPase wasn't suspected until 1967 when Nishi and Koketsu (1967) reported that ouabain had a highly specific effect on the slow inhibitory postsynaptic potentials of sympathetic ganglia, suggesting the involvement of an electrogenic Na^+ pump in the process. Around that time, evidence supporting the regulatory role of catecholamines on the Na^+/K^+-ATPase started accumulating. Norepinephrine stimulated the enzyme's activity in brown adipose tissue (Herd *et al.,* 1970), myelin fraction of cat brain (Iwangoff*et al.,* 1974), skeletal muscles (Cheng *et al.,* 1977), and rat cortex (Wu *et al.,* 1979). A similar positive effect was also observed in rat brain synaptic membrane (Clausen& Formby, 1967) and resting skeletal muscles (James *et al.,* 1999) incubated with epinephrine, but not in dopamine- treated adult rat jejunal cells during high salt diet (Vieira-Coelho*et al.,* 1998).

Different mechanisms of action were proposed for the effects of catecholamines on the Na^+/K^+-ATPase. Short-term treatment of alveolar cells with β-agonists resulted in a cAMP-mediated upregulation in Na^+/K^+-ATPase gene expression and a corresponding increase in its activity(Minakata*et al.,*1998).The binding of norepinephrine to the rat brain α1-adrenergic receptors, on the other hand, elevated cytosolic Ca^{2+} and activated calcineurin, a Ca^{2+} /calmodulin dependent phosphatase, to dephosphrylate and stimulate the Na^+/K^+-ATPase (Mallick*et al.,*2000).Finally, dopamine inhibition of the Na^+/K^+-pump was found to be associated with an increased endocytosis of α-subunits from the plasma membrane in a PKC, PI3K (Chibalin*et al.,*1998), and AP-2 mediated process (Ogimoto*et al.,*2000).

a. Na^+/K^+-ATPase properties

The Na^+/K^+-ATPase , also known as the Na^+/K^+-pump , is ubiquitously expressed and essential for the survival of all animal cells (Vasilets & Schwarz ,1994). It belongs together with the Ca^{2+} ATPase of the sarcoplasmic reticulum and the H^+/K^+ ATPase of the stomach (Koksoy, 2002), to the P-type family of ATPases , that undergo a phosphorylation-dephosphorylation cycle, accompanied by a change in conformation, to actively transport ions across the plasma membrane (Jorgensen *et al .,* 2003). For every ATP it hydrolyzes, the Na^+/K^+-ATPase pumps three Na^+ out and two K^+ into the cell ,thereby creating a Na^+ and K^+ electrochemical gradient across the membrane (Therien &Blostein , 2000) that is essential for the maintenance of cell membrane potential, cell volume, cell pH , Ca^{2+} , and Cl^- levels via Na^+/H^+ , Na^+/Ca^{2+} , and $Na^+/Cl^-/K^+$ exchangers

respectively. These gradients also drive the Na^+- dependent secondary transport of some nutrients like glucose and amino acids across the cell membrane of intestinal and renal epithelial cells (Lopina O.D., 2001). Thus the proper regulation of the Na^+/K^+-ATPase is essential for the proper functioning of the cell.

The Na^+/K^+-pump is a large hetero-oligomer composed of two polypeptide chains joined together by non-covalent bonds: a 110 KD α subunit composed of 10 transmembrane domains with N and C-terminal chains facing the cytosol and two large intracellular loops, and a smaller 55 KD β subunit composed of a single transmembrane domain with a short cytoplasmic N-terminus and a long glycosylated extracellular C-terminus (Lopina O.D., 2001). The subunit is the catalytic subunit with transient phosphorylation sites and binding sites for cardiac glycosides, ions (Na^+, K^+.Mg^{2+}), and ATP. It exists in four different isoforms (α1, α2, α3, α4) differentially expressed in tissues and species (Koksoy, 2002). The β-subunit is thought to play a supportive role. Its oligomerization with the alpha subunit seems to be important for the function of the enzyme and for the protection of the α-subunit from degradation. It also acts as a chaperon to ensure the proper folding and delivery of the α-subunit to the membrane, and is thought to participate in the formation of the binding sites of the ligands as well. It exists in 3 isoforms (β1, β2, β3) with differential expression among species and tissues.

Different combination of α and β isoforms are present in different tissues. . The α1β1 dimer is almost ubiquitously expressed in all cells (Lopina, 2001).

b Na^+/K^+-ATPase regulation

In general, the Na^+/K^+-ATPase is subject to two types of activity modulations: Long term regulation that interferes with the biosynthesis and degradation of the α and β subunits, and short term regulation which affects the kinetic behavior of the enzyme and its translocation to the cell membrane from intracellular stores (Therien*et al.,*2000). Post-translational phosphorylation, by PKA, PKC, and PKG, is a well understood example of the rapid short-term regulation of the sodium pump activity.

PKA phosphorylation site at Ser 943 was first identified in the C-terminal of the rat renal Na^+/K^+--ATPase α1 subunit (Feschenko*et al.,* 1995).Despite the fact that it was shown later on, to be conserved in all tissues (Lopina, 2001), the effects of its phosphorylation in various cell types varies between activation (Cornelius & Logvinko, 1996), inhibition (Borteorello*et al.,* 1991), or no change at all (Feschenko*et al.,* 1995). This discrepancy can be explained by the isoform specific effects of PKA whereby transfection of Hela cells with different α isoforms in the presence of dibutyryl cAMP (PKA activator) resulted in the direct phosporylation of all isoforms but in the activation of α3 subunit and the inhibition of α1and α2 isoforms (Blanco *et al.,* 1998).

In addition to direct phosphorylation , more complex mechanisms of action were proposed for PKA regulation of the Na^+/K^+-ATPase that include the activation of mediators such as the PLA2 / eicosanoid synthesis pathway (Satoh *et al.,*1993) and protein-phosphatase inhibitors(DARPP-32/Inhibitor I) (Aperia*et al.,*1994)

PKC is another potent regulator of the Na^+/K^+-ATPase capable of activating (Greene *et al.,* 1986&Lahaye *et al.,* 1998) or inhibiting (Cheng *et al.,* 1997 &Blanco *et al.,* 1998) the

enzyme in a tissue and species dependent manner. Various phosphorylation sites of the α1 subunit by PKC have been identified in various species. These sites include Ser11, Ser 16, Ser18, and Ser23 in rat (Vasilets, 1997&Efendiev*et al.,* 2000), Ser11 for sheep (Beguin*et al.,* 1994), pig and dog (Feschenko & Sweadner, 1995), and Thr 15 and Ser 16 in *Bufomarinus* (Beguin*et al.,* 1994). Whether their direct phosphorylation by PKC alters the Na^+/K^+-ATPase activity remains unclear; nonetheless, experiments have shown that PKC-dependent phosphorylation at specific residues can alter the ATPase's affinity for Na^+ (Feraille*et al.,* 2000), K^+, and Mg^{2+} ions (Ramnanan & Storey, 2006), and promote its endocytosis (Dada & Sznajder, 1999) and exocytosis (Bertorello *et al.,* 1999) from and to the cell membrane.

An indirect modulation of the Na^+/K^+-ATPase by PKC is also possible via activation of the PLA2/eiconsaid synthesis pathways (Xia *et al.,* 1995), as in the case with PKA, or the stimulation of NO/cGMP/PKG pathways (Chen *et al.,* 2005).

PKG is another kinase reported to phosphorylate the α 1 subunit of Na^+/K^+-ATPase purified from the dog, sheep , pig, rat kidney , and Xenopus Oocyte . Little is known about the specific phosphorylation residues, but immunoassays identified the phosphorylation sites to fall in the intracellular loop of α- subunit between the 35 KDa N-terminal and 27-KDa C-terminal portions (Fotis*et al.,* 1999).

PKG regulatory effects on the Na^+/K^+-ATPase are well established and tissue-dependent. PKG was reported to inhibit the Na^+/K^+-ATPase in alveolar cells (Guo*et al.,*1998), skeletal muscles(Li&Sperelakis,1993), brain (Pontigia*et al.,*1998), and colon (Schreiner *et al.,*1980), but to stimulate the enzyme in pulmonary smooth muscles (Tamaoki *et*

*al.,*1996), mammalian arteries (Ferrer *et al.,*1995), Purkinjie neurons (Nathanson*et al.,*1995), and duck salt gland (Stewart & Sen,1981).

Whether PKG, however, exerts its effects in a direct or indirect manner remains to be elucidated.

B. Prostaglandins

Prostaglandins are a group of biologically active lipid mediators that belong to the eicosanoid family of fatty acids (Lands, 1979), and are produced and secreted by almost all cells of the body (Park *et al.,* 2006). They act as homeostatic modulators under physiological conditions (Miller, 2006), or disease markers and causative agents in pathological settings (Dubious *et al.,* 1998, Harris *et al.,* 2002). Arachidonic acid, a polyunsaturated fatty acid, is released from the cell membrane by the enzyme phospholipase A2, and is oxidized by the cycoloxygenase enzyme (COX) to produce the unstable intermediate PGG2 .PGG2 is subsequently reduced, by the same COX, to the prostaglandin precursor PGH2. Several terminal enzymes will catalyze the formation of the different types of prostaglandins (PG) including prostaglandin E2 (PGE2) by PGE2 synthase (Park*et al.,* 2006).

Two isoforms of the COX enzyme were identified: COX-1 and COX-2. Both having similar function and enzymatic activity as each is capable of catalyzing the two sequential oxygenation and reduction reaction in PG synthesis; nonetheless, they are coded by distinct genes resulting in their differential mode of expression. While COX-1 is reported

to be constitutively expressed, Cox-2 expression is induced by certain cytokines, growth factors and other signaling molecules (Vane, 1998).

PGE2 is among the most well studied prostaglandins and is believed to be the most abundant in the human body (Park*et al.,* 2006). It exerts its effects by binding to one or more of its E-prostanoid G-protein coupled receptors (EP1, EP2, EP3, EP4) (Bos *et al.,* 2004) to initiate signaling cascades that will result in a specific altered cellular response. Each receptor subtype is associated with different types of G-proteins and thus acts through a distinct set of second messengers. While the binding of PGE2 to Gq coupled-EP1 receptor induces the activation of PKC and the elevation of cytosolic Ca^{2+} as a result of the hydrolysis of PIP2 by PLC into DAG and IP3 respectively (Herbert *et al.,* 1990), its binding to EP2 and EP4 receptors activates Gs which stimulate adenylate cyclase (AC) to produce cAMP, leading to PKA activation (Nakao*et al.,* 1989). EP3 receptor, on the other hand, acts to inhibit AC via the activation of Gi (Sonnenburg *et al.,* 1990).

1. PGE2 and the Na^+/K^+-ATPase

A downstream target of prostaglandins is the Na^+/K^+-ATPase. Satoh *et al.* (1992) reported that arachidonic acid pathway products, especially the cycoloxygenase metabolites, may contribute to the indirect inhibition of the renal Na^+/K^+-ATPase through the PLC-PKC route (Ominato*et al.,* 1996). In general, PGE2 appears to act as a negative modulator of the pump in a variety of tissues, despite the fact that a few studies suggest a stimulatory effect on the pump (Kreydiyyeh *et al.,* 2006). An increase in PGE2, associated with a decrease in cAMP, mediated angiotension II inhibitory effects on Na^+/K^+-ATPae and

water absorption in rat jejunum (Jin *et al.,* 1998) .PGE2 was also shown to reduce Na^+/K^+-ATPase protein expression in LLCPK1 (Kreydiyyeh *et al.,* 2004) ,cardiomyocytes (Skayian & Kreydiyyeh, 2006) ,and HepG2 cells (Kreydiyyeh *et al.,*2007). Incubation of rat hippocampus ,both in vivo and in vitro, with PGE2 for 30 min led to a dose dependent decrease in the Na^+/K^+-ATPase activity attributed to the PKA and PKC- dependent Ser943 phosphorylation of the α subunit (Oliveria *et al.,*2009).

2. PGE2 and catecholamines

Prostaglandins are another group of intracellular molecules modulated by catecholamines. Norepinephrine was found to stimulate PGE2 synthesis in a variety of tissues including spleen (Bruckner-Schmidt *et al.,* 1981), kidney (Needleman *et al.,* 1974), brain (Seregi*et al.,* 1982, Birkle*et al.,* 1981), rabbit and bovine Irides (Yousufzai *et al.,* 1983), and sympathetic neurons (Sherbourne *et al.,* 1992).

Only a few studies addressed the mechanism of action underlying norepinephrine-induced synthesis of PGE2, and thus it is still poorly understood. Nonetheless, α adrenoceptor-dependent Ca^{2+} increase/PLA2 activation and β adrenoceptor –dependent cAMP production were shown to be two possible routes in rat splenic pulpa (Bruckner-Schmidt *et al.,*1981) and primary rat microglia (Schachetzki *et al.,*2010) respectively.

C. Nitric Oxide

Nitric oxide ($NO^{\cdot}$) is a small 30Da biosynthetic molecule (Brodsky *et al.,* 2001) with important physiological roles both in health and disease. Its pharmacological effects

extend to a variety of organ systems to modulate processes such as neuronal excitability (Erdemli & krnjevie, 1995), arterial vasodilation (Furchgott*et al.,* 1980), smooth muscles relaxation (Desai *et al.,* 1991), and immune defense reactions (Xie *et al.,* 1996; Nathan *et al.,* 2000). Nonetheless, when present in excessive amount , NO can lead to neurotoxicity (Dawson *et al.,* 1993), inflammation (Vane *et al.,* 1994), circulatory and haemorrhagic shocks (Wright *et al.,* 1992), just to name a few.

Intracellular NO is released as a by-product from the oxygenation of L-arginine to L-citrulline by nitric oxide synthase (NOS), in the presence of calcium/calmodulin complex (CAM) and NADPH (Nathan, 1992). Three genetically distinct NOS isoforms (Nakane *et al.,* 1993, Marsden et *al.,* 1992; Geller *et al.,* 1993) were identified based on their localization, expression, and Ca^{2+} dependency. nNOS and eNOS , predominate in neural and endothelial tissues respectively (Wu,1993,Venugopol *et al.,* 2002), are generally constitutively expressed (Forstermann *et al.,*1998) and are regulated by Ca^{2+} (Michel *et al.,* 1997). iNOS , however, appears to be expressed only upon demand, in response to certain cytokines in almost all nucleated cells (Griffith & Stuehr,1995), and was found to possess a Ca^{2+} independent activity (ladecola *et al.,*1995).

Unlike other signaling molecules , no membrane receptors have yet been identified for NO due to its small size and solubility in both water and lipids (Pacher *et al.,*2007), NO can passively diffuse in and out of the cells to act in a paracrine or autocrine manner (Shah&MacCarthy,2000). Once in the cytosol, NO activates the soluble guanylyl cyclase to trigger the cGMP –dependent and PKG-dependent signaling pathways that mediate its various cell-specific effects (Downey *et al.,* 2007).

In general, the activity of all NOS is limited by the availability of their substrates and cofactors, their localization, and their interaction with the CAM protein. Nonetheless, these enzymes are more acutely regulated by several different pre- and post translational mechanisms.

The majority of the iNOS regulation seems to occur at the level of its expression as revealed by DNA sequencing experiments which identified several promoter regulatory elements (Chartrain*et al.,*1994 , Janssen-Heininger*et al.,* 2000) specific to cytokine – induced transcription factors ,such as NF-kB,AP-1 ,HIF, and STAT1-α just to name a few (Kleinert *et al.,*2004) . Furthermore, iNOS mRNA was shown to be unstable in the absence of HuR, an mRNA binding protein (Rodriguez-Pascual *et al.,* 2000). There is accumulating evidence, however, that iNOS activity can also be controlled and stabilized via protein phosphorylation either directly by ERK on Ser745 (Zhang *et al.,* 2007) and Src on Y151 (Hausel*et al.,* 2005) and Y1055 (Tyryshkin*et al.,* 2010), or indirectly by PKC-dependent MAPKs phosphorylation and subsequent activation of NF-kB (Wen *et al.,* 2011).

Similarly, a variety of protein kinases were identified as modulators of nNOS and eNOS activity. Phosphorylation of eNOS at S1179 by PKB (Dimmeler*et al.,*1999), PKA (Boo *et al.,*2002),PKG (Butt *et al.,*2000),AMPK (Chen *et al.,*1999),CAMKII (Fleming *et al.,*2001) , and at S116 by PKC (Kou *et al.,*2002) was shown to increase the activity of eNOS . On the other hand, its phosphorylation at S635 by PKA (Boo *et al.,* 2002), and T497 by AMPK (Chen *et al.,* 1999) and PKC (Fleming *et al.,* 2001), decreased the activity of eNOS.

An inhibitory role for tyrosine kinases such as Src was proposed in the regulation of eNOS. Nonetheless, little is known about the specific sites and its effect on the activity (Boo & Jo, 2003).

The effect of phosphorylation of nNOS is less studied with various phosphorylation sites for PKA (Brune&Lapetina,1991), PKG (Dinerman *et al.,*1994), PKC ,and CAM – dependent kinases identified (Nakane *et al.,*1991). The significance of such a phosphorylation is still controversial with scarce evidence supporting a negative role for these kinases in the regulation of nNOS activity (Nakane *et al.,* 1991&Dinerman*et al.,* 1994).

1. Nitric oxide and the Na^+/K^+-ATPase

Nitric oxide modulates the Na^+/K^+-ATPase activity in a tissue-dependent manner. NO inhibited the pump in M441 pulmonary epithelial cells (Althaus *et al.,*2011) ,Porcine cerebral cortex (Sato *et al.,*1997), and lipopolysaccharide –treated guinea pig liver and kidney cells(Seven *et al.,*2005&Cimen *et al.,*2004). Furthermore, NO/cGMP pathway was found to act as a negative modulator of the pump in alveolar type II cells (Guo *et al.,*1998) , opossum kidney monolayers (Liang &Knox,1999), and Angiotensin II and carbachol incubated rat proximal tubule (Zhang & Mayeux,2001; Hakam&Hussain,2006) and choroid plexus (Ellis *et al.,*2000) respectively. Similarly NO, in MTAL cells overexpressing NOS, inhibited the transcription of the α1 subunit of the Na^+/K^+-ATPase and consequently decreased its activity (Kone & Higham, 1999).

On the other hand, Positive regulatory effects of NO on the Na^+/K^+-ATPase were observed in cardiac myocytes (White *et al.,*2008) , proximal segment of rat trachea (Akici*et al.,*2000) , and SH-sY5Y human neuroblastoma cells (Inada *et al.,*1995).

2. Nitric Oxide and catecholamines

Since both NO and catecholamines act as potent effectors of the cardiovascular system, scientists suspected a possible crosstalk between NO and adrenergic pathways. Indeed , several publications supported this notion and established the regulation of NO by catecholamines via the different subtypes of the α (Jones *et al.,*1993,Thorin *et al.,*1998) and β (Gauthier *et al.,*1998, Ferro *et al.,*2004 ,& Chen *et al.,*2007) adrenoceptors.
The direction of this regulation and its underlying signaling players varied between different tissues. While epinephrine activated eNOS in BAEC cells via β3AR stimulation and the sequential activation of Rac1,PKA, and Akt (Kou *et al.,*2007), it decreased the production of NO in macrophages through a β1/ β2 ARs (Sigola *et al.,*2000) , IL-10, and TNF-α mediated pathway(Zinyama*et al.,*2001).Likewise, the binding of norepinephrine to its β-ARs and consequent increase in cAMP/PKA levels enhanced NOS activity in cardiac myocytes (Kanai *et al.,*1997) and macrophages(Chi *et al.,*2003) , but not in hepatocytes (Collins *et al.,*2001).

D. G-proteins

Both PGE2 and epinephrine act via guanine nucleotide binding proteins, known as G-proteins, which are heterotrimers of three subunits, α, β, and γ, and act as signal mediators

of extracellular chemical and physical stimuli. They are cyclically regulated by the association of the α-subunit with GTP/GDP. The binding of the receptor to its specific ligand will trigger the G-protein to exchange the α-bound -GDP with GTP. The active α-GTP subunit will dissociate from the βγ complex and both will further interact with downstream effectors to transduce the initial signal .The hydrolysis of the GTP back to GDP will initiate the deactivation process upon which the three subunits will associate to render the G-protein inactive again (Hepler & Gilman,1992).

G-proteins are divided into different families based on structural and sequence similarities. Gs is classified as stimulatory G-protein due to its ability to stimulate adenylyl cyclase (AC) enzyme responsible for the cyclization of ATP to cAMP which in turn will activate protein kinase A (PKA) (Gilman, 1987).Gi or inhibitory G-proteins, however, act opposite to Gs to inhibit AC and downregulate cAMP (Taussig *et al.,* 1993).Interestingly, both α and βγ subunits of Gi were shown to communicate signals. While Gαi is mainly responsible for AC inhibition (Taussig *et al.,* 1993), Gβγi are capable of directly regulating effectors such as PLC-β, K^+ channels, PI3K, and even AC (Neves *et al.,* 2002).

Gq and Go, although of distinct categories, work via the same signaling molecules which were first identified to be the classical pathway for calcium-mobilizing hormones (Ghosh *et al.,* 1996).

Both Gαq/o will stimulate PLC to cleave PIP2 into IP3 and DAG. IP3, consequently, will bind and open IP3-sensitive Ca^{2+} channels in the endoplasmic reticulum to induce the

release of Ca^{2+} from the ER lumen. DAG, on the other hand, will stay bound to the cytosolic face of the cell membrane and will recruit and activate PKC (Lukas, 2004).

A less defined family of G-proteins is the G12 / G13 family; despite extensive studies, little is known about the signaling effects of G12 and G13 proteins. Nonetheless, it was reported that Gα12 might be involved in the activation of c-Src, PKC, and members of the MAPK family (Neves *et al.,* 2002). Similarly, Gα13 was shown to activate Rho by directly binding to and stimulating its guanine –nucleotide exchange factor that promotes the hydrolysis of Rho-GDP into Rho-GTP (Neves *et al.,* 2002).

E. Mitogen Activated Protein Kinases (MAPKs)

Mitogen activated protein kineases (MAPKs) are a family of evolutionary conserved serine/threonine kinases that serve as focal points in several cellular responses such as cell proliferation (Zhang & Liu,2002),survival (Bonni *et al.,*1999), differentiation (Laprise *et al.,*2002) , motility(Krueger *et al.,*2001),and immunity (Garcia-Garcia *et al.,*2008). To date, three MAPK pathways have been identified in eukaryotic cells based on the respective classification of the MAPKs into three different subgroups: extracellular signal-regulated kinases (ERK), c-jun N-terminal kinases, and p38 kinases (Cargnello & Roux, 2011). Each pathway is composed of a set of three sequentially acting kinases: MAPKK kinase (MAPKKK), MAPK kinase (MAPKK), and MAP kinase (MAPK) (Hommes*et al.,* 2003). The activation of MAPKKKs by their phosphorylation or interaction with GTP-binding proteins of the Rho/Ras family, will allow them to phosphorylate and activate MAPKKs which, in turn, will phosphorylate MAPKs at their

two unusual sites threonine and tyrosine. Active MAPKs can then phosphorylate cytoplasmic or nuclear proteins to modulate their activity (Pearson *et al.,* 2001).

1. ERK properties

The ERK module includes Raf-1, A-Raf, or B-Raf as MAPKKK, MEK1 and MEK2 as MAPKK, and ERK 1 and ERK2 isoforms as MAPK (Cargnello & Roux,2011).ERK proteins were shown to be activated in response to cytokines (Lejeune *et al.,*2002), osmotic stress (Kim *et al.,*2000), cytoskeleton disorganization (Kawamura *et al.,*2003), and the stimulation of receptor tyrosine kinases (Boulton *et al.,*1991) and GPCR (Sugden&Clerk,1997).

ERK modulation by GPCR depends on the nature of their cognate G-proteins. Gαs-induced cAMP was shown to decrease or increase the activity of ERK1/2 in a cell-specific manner (Zheng *et al.,* 2000, Norum *et al.,* 2003& Keiper *et al.,* 2004). ERK inhibition by cAMP is thought to be due to the decreased interaction between c-Raf and Ras following c-Raf phosphorylation at Ser 43 and 621 by PKA (Yip-Schneider *et al.,* 2000; Volonte & Greene, 1990). On the other hand , elevated cAMP can result in ERK stimulation by either activating Ras , Rap-1/B-Raf via the guanine nucleotide exchange factor EPAC(Vossler *et al.,*1997; Daakra *et al.,*1997), or Src (tyrosine kinase) to phosphorylate and activate c-Raf (Maudsley *et al.,*2000).Paradoxically, cAMP appears to play a less important role in heart where β-adrenergic stimulation of ERK is more likely dependent on the elevation of intracellular Ca^{2+} instead (Bogoyvitch *et al.,*1996).

In vivo studies indicated that Gαi coupled receptors can increase the activity of ERK by one of two mechanisms. By down- regulating cAMP, Gαi will relieve the inhibitory effect of PKA on c-Raf and consequently render ERK active (Radhika & Dhanasekaran, 2001). Alternatively, incubation with βγ sequestering peptide (Lopez-Ilasaca *et al.,* 1997) and the overexpression of the βγ subunit (Della Rocca *et al.,* 1999) showed that this complex is necessary for Gi –induced ERK stimulation, probably via a pathway involving PI3K, PLC, and/or Src (Daub *et al.,* 1996; Li *et al.,* 1998). Experiments on transfected HEK-293 cells expressing Gi-coupled α2 –adrenergic receptors led to the conclusion that the released βγ subunit activates PLC to induce an IP3-dependent increase in cytosolic Ca^{2+}. Ca^{2+} elevation will trigger a Ca^{2+}-calmodulin mediated stimulation of Pyk2 kinase followed by the subsequent activation of Src, Ras, and the MAPK cascade (Lev et al., 1995; Dikic et al., 1996; Luttrell *et al*., 1996; Della Rocca *et al*., 1997). In addition, others reported that the βγ subunit can act to induce the tyrosine phosphorylation of the adaptor protein Shc which in turn will associate with Grb2 and SOS to increase GTP-binding to Ras. GTP-Ras can then activate Raf and the subsequent ERK module (Kranenburg *et al.,* 1999b).

Analysis of Gq Erk –mediated signaling revealed that Gαq can stimulate ERK by different pathways. Gαq coupled muscarininc receptors in Cos-7 and CHO cells activate ERK via PKC-c Raf signaling axis (Hawes *et al.,* 1995). In contrast, Lysophospholipid receptors ERK activation is mediated by calcium-calmodulin complex, Pyk2 kinase, Src, and Ras (Dikic *et al.,*1996). Furthermore, α1-adrenergic signaling in HEK293 cells appears to

employ both PKC and Ca^{2+}-calmodulin pathways for the induction of ERK (Della Rocca et al., 1997).

In contrast to other G-proteins, little is known about G12/13 MAPK modulation except that it seems to act as negative regulator to attenuate ERK's activity (Voyno-Yasenetskaya *et al.,* 1996). Although the underlying mechanism of such attenuation is not fully understood, it is believed that PP5, a Raf-1 phosphatase, might be involved (Von Kriegsheim *et al.,* 2006).

2. ERK and Na^+/K^+-ATPase

MAP kinases have been implicated as regulators of the Na^+/K^+-ATPase .Dopamine like receptor activation , in kidney proximal tubule cells, increased Na^+/K^+-ATPase activity by means of the ERK –pathway (Narkar *et al.,*2002).Angiotensin II mediated ERK-induction upregulated α1 subunit gene transcription and consequently Na^+/K^+-ATPase activity in vascular smooth muscle cells (Isenovic *et al.,*2004). Moreover, ERK activation by C-peptide and fibroblast growth factor resulted in the phosphorylation of the α subunit and the short-term stimulation of the Na^+/K^+-ATPase in human renal tubular cells and alveolar epithelial cells respectively (Upadhyay *et al.,* 2003, Zhong *et al.,* 2004).

3. ERK and PGE2

Different studies reported MAPK as a potent effector of COX-2/PGE2 axis in response to various stimuli in several cell lines: IL-1β stimulated fibroblast -PGE2 synthase and endometrial COX-2 through ERK and p38 MAPK respectively (Kida *et al.,* 2005; Huang

et al., 2013). TGF-β –induced ERK/p38 MAPK/PI3K pathway increased COX-2 expression and PGE2 levels in HMC cells (Rodriguez-Barbero *et al.,* 2006).NSAID pro-apoptotic effects in colorectal carcinoma cells resulted from ERK-mediated COX-2 overexpression (Elder *et al.,* 2002), and the stimulation of ERK signaling in response to ceramide up-regulated COX-2 synthesis via c-Jun and cAMP in mammary epithelial cells (Subbaramaiah *et al.,* 1998).

Nonetheless, accumulating evidence indicated that PGE2 can also itself act upstream of ERK to control its activity, as observed in rabbit corneal epithelial cells whereby EGF-induced PGE2 activated PKA to inhibit Raf-1 and ERK cascade (Kang *et al.,* 2000). In contrast , PGE2 up-regulated ERK in human colon cancer cells (Sheng *et al.,*1998), lung carcinoma cells via a Ca^{2+} dependent route (Krysan*et al.,*2005), dendritic cells through cAMP/PKA/PI3k pathway (Yen *et al.,*2011), and endothelial cells via PKC (Corti *et al.,* 2013).

4. ERK and NO

Similar to PGE2, there exists a reciprocal relationship between ERK and NO, probably as a part of a feedback loop regulatory mechanism.

While still controversial, Ser 114 residue, in the oxygenase domain of eNOS, was identified as a potential target site for ERK phosphorylation (Fleming *et al.,* 2003). Whether such a phosphorylation would affect eNOS activity remains to be elucidated. Nonetheless, Increasing ERK, in response to VEGF, in glomerular endothelial cells, was shown to phosphorylate eNOS at Ser 1177 and up-regulate its activity (Fliers *et*

*al.,*2005).Chrestensen *et al.* further demonstrated that ERK-2 can strongly bind and inhibit eNOS (2012). ERK regulation of NOS can also take place at the transcriptional and post-transcriptional levels as observed in endotoxin-stimulated glial cells (Bhat *et al.,* 1998). In addition to functioning as an upstream regulator, ERK was shown to mediate some of the cellular effects of NO. NO induced ERK- activation promoted cell survival and dedifferentiation of chondrocytes (Kim *et al.,*2002).The addition of NOS inhibitor prevented hypoxia-dependent ERK phosphorylation in cortex of newborn piglets (Mishra *et al.,*2004), suggesting that this phosphorylation is NO mediated . Moreover, the NO/cGMP axis was shown to promote synaptic plasticity by the regulation of ERK and ERK-induced gene expression at pre- and postsynaptic sites of amygdala and thalamus nuclei following long term potentiation (Ping & Schafe, 2002).

CHAPTER III

MATERIALS AND METHODS

A. Materials

Dulbecco's Minimal Essential Medium (DMEM) with 4500mg glucose/L and pyridoxine HCl , Fetal Bovine Serum(FBS), Trypsin-EDTA , Penicillin/Streptomycin(PS) ,10x Phosphate Buffered Saline (PBS) without calcium and magnesium , (-)-Epinephrine, L-Ascorbic Acid, N^6,2′-O-Dibutyryladenosine 3′,5′-cyclic monophosphate sodium salt (dbcAMP) , Adenosine 5′-triphosphate disodium salt(ATP),

Ouabain, Prostaglandin E2 (PGE2) , Indomethacin , DL-Propranolol , Butoxamine-HCL ,Prazosin , and Yohimbine were purchased from Sigma,Chemical Co,St. Louis Missouri ,USA.

Phorbol-12-myristate-13-acetate (PMA), PD98059, and Calphostin C were purchased from CALBIOCHEM, San Diago, USA.

Glyco-SNAP1, Carboxy-PTIO, and SC 19220 were purchased from Santa Cruz Biotechnology, CA, USA.

Protease inhibitor cocktail tablets were bought from Boehringer Mannheim, Germany.

Biorad assay protein reagent was purchased from Biorad, CA, USA.

The human colon carcinoma cell line (CaCo-2) from a Caucasian male was bought from American Type Culture Collection (ATCC), VA, USA.

All other chemicals were purchased from Sigma, Chemical Co, St. Louis Missouri.

B. Methods

1. Cell Culture of CaCo-2 cells

CaCo-2 cells were used at passages 25-32. They were grown, at a density of 120,000 cells/ml , on 100mm culture dishes in DMEM containing 4500 mg L-1 Glucose, sodium pyruvate, 1% Penicillin (100 µg mL^{-1}), streptomycin (100 µg mL^{-1}), 10% FBS, in a humidified incubator (95% O2, 5% CO2) at 37°C. Cells were always treated at 90-100% confluence.

2. Treatment of CaCo-2 cells

a. Effect of Epinephrine on the activity of the Na^+/K^+-ATPase

The effect of epinephrine on the pump was studied by treating the cells with 0.5mM epinephrine dissolved in 0.5M of ascorbic acid. The positive and negative control groups were incubated with and without ascorbic acid respectively.

b.Protein Extraction and Determination

At the end of the treatment period, cultured cells were washed twice with 1x PBS solution (5.16g NaCl, 1.5g Na_2HPO_4, 1.09 g KH_2PO_4 in 1L H_2O ; pH 7.3) and lysed with 300 µl Histidine lysis buffer (9.9ml of 150mM Histidine (pH7.4), 400µl protease inhibitor(1 tablet/ 2 ml H_2O), 100µl Triton-X(1mg/ml H_2O) , then scraped , and homogenized at 4°C in a polytron at 22,000rpm . Proteins were quantified colorimetrically at a wavelength of 595nm using the Bradford Biorad assay.

c. Na^+/K^+-ATPase Activity Assay*:*

Protein concentration of each sample was adjusted to 0.5µg/µl using histidine buffer (150mM, pH 7.4). To a sample volume of 65µl, 17 µl of 1% saponin and 13µl of phosphatase inhibitor cocktail (300µl of 200mM glycerophosphate, 300µl of 200mM pyrophosphate, 400µl H_2O) were added.

The mixture was incubated at room temperature for 30 minutes.

Aliquots from each sample were then withdrawn and suspended in a buffer containing the substrates NaCl (1240mM), KCl (200mM), $MgCl_2$ (40mM), and ATP (30mM) and incubated in presence and absence of ouabain (15mM) as shown in the table below, for a period of 30 minutes at 37°C .

	µl	µl
NaCl (1240mM)	10	10
KCl (200mM)	10	10
$MgCl_2$(40mM)	10	10
Histidine (150mM,pH7.4)	20	20
H_2O	30	0
Homogenate	12	12
ATP(30mM)	10	10
Ouabain(15mM)	0	30
Total Volume	102	102

The reaction was then stopped by the addition of 10 µl of 50% tricholoacetic acid solution to each sample

The samples were then spun at 14000 rpm for 5 minutes and the amount of inorganic phosphate liberated in the supernatant was measured colorimetrically, in the presence of Ferrous sulfate –molybdate reagent (0.5g Ferrous sulfate, 1 ml Ammonium molybdate (0.1g/L of 10N H_2SO_4) , 9ml H_2O) at a wavelength of 750nm. Each well contained 100 µl of the supernatant and 80µl of ferrous sulfate molybdate reagent.

3. The signaling pathway

a. Determination of the type of adrenergic receptors involved

The type of adrenergic receptors mediating the effect of epinephrine on the pump was determined by pre-treating the cells , 20 minutes prior to the addition of epinephrine , with the following antagonists: 0.1 mM Yohimbine (α2 adrenergic antagonist) or 0.03mM Propranolol (non-selective β-adrenergic blocker).

The nature of the G-protein coupled to the respective adrenergic receptor was determined by incubating the cells for 20 minutes RpcAMP (30µM), a PKA inhibitor. The vehicle was always added to the control in the same amount and for the same time.

b. Identifying the signaling mediators involved

The involvement of PGE2,NO, PKC, and MAPK/ERK was suspected and investigated by pre-treating the cells, 20 min prior to the addition of epinephrine , with their respective inhibitors : indomethacin (100µM) (COX-inhibitor) , PTIO (30µM) (NOS inhibitor) , calphostin C(50nM, dissolved in DMSO) (PKC inhibitor),and PD98059 (50 µM dissolved in DMSO ,MEK/ERK inhibitor)

To investigate further the role of PGE2, NO, and PKC in the modulation of the Na+/K+ ATPase activity, the cells were treated with exogenous PGE2 (1nM) of 2µM of Glycol-SNAP1(NO generator) , and PMA(100nM, dissolved in DMSO,(PKC activator).
Since PKC is activated by EP1 receptors, the possibility that PGE2 might be acting through these receptors was examined by pre-incubating the cells, 20 minutes prior to the addition of epinephrine, with the EP1 selective antagonist SC19220 (100µM dissolved in DMSO). The vehicle was added to the control at the same concentration.

c. Locating the different mediators in the pathway

Locating the mediators with respect to each other was determined via a similar procedure as the previous treatments; the location of PKC was investigated by pre-treating the cells with indomethacin and PTIO for 20minutes, prior to the addition of PMA, an activator of PKC. Similarly, to determine the location of PGE2 the cells were treated with exogenous PGE2 in the presence of PTIO or Calphostin C.

4. Statistical Analysis

Results are reported as means±SEM and are tested for statistical significance by a one-way Analysis of Variance (ANOVA) followed by Tukey-Kramer multiple comparisons test using Instat and Excel Softwares. The results were considered significant at $P<0.05$.

CHAPTER IV

RESULTS

A. Effect of epinephrine on the activity of the Na^+/K^+-ATPase

CaCo-2 cells treated with 0.5mM of epinephrine (dissolved in ascorbic acid) for 20 minutes exhibited a 50 % decrease in the activity of the Na^+/K^+-ATPase. Ascorbic acid however, added at a concentration of 0.5M had no significant effect on the activity of the pump as seen in figure (1).

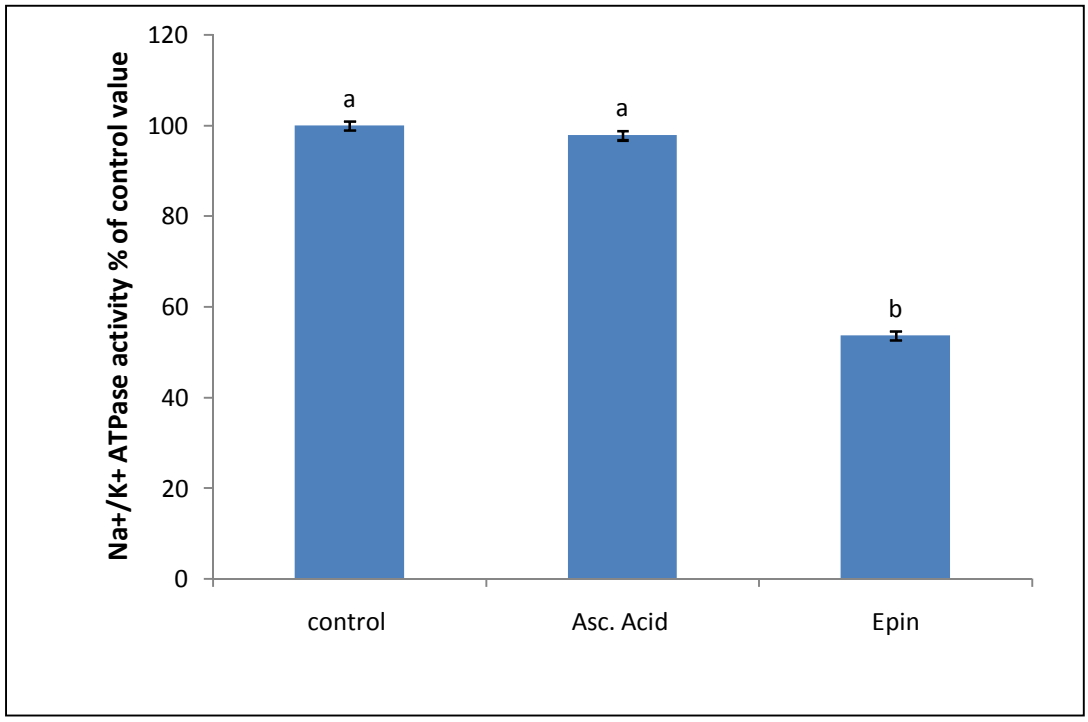

Figure 1: Effect of Epinephrine and Ascorbic Acid on the activity of Na^+/K^+-ATPase in CaCo-2 cells. Values are means±SEM. N=20. Bars not sharing a common superscript are considered significantly different from each other at $P<0.01$.

B.Alpha-2 adrenergic receptors mediate the effect of epinephrine on the pump

The inhibitory effect of epinephrine on the Na^+/K^+-ATPase persisted when the cells were pre- incubated with of 0.03 mM Propranolol (non selective β-adrenergic blocker) , but was no longer apparent in the presence 0.1mM Yohimbine , the selective α2- adrenergic antagonist (fig2a,b,), suggesting that epinephrine exerts its effect by exclusively binding to its α2-adrenergic receptors.

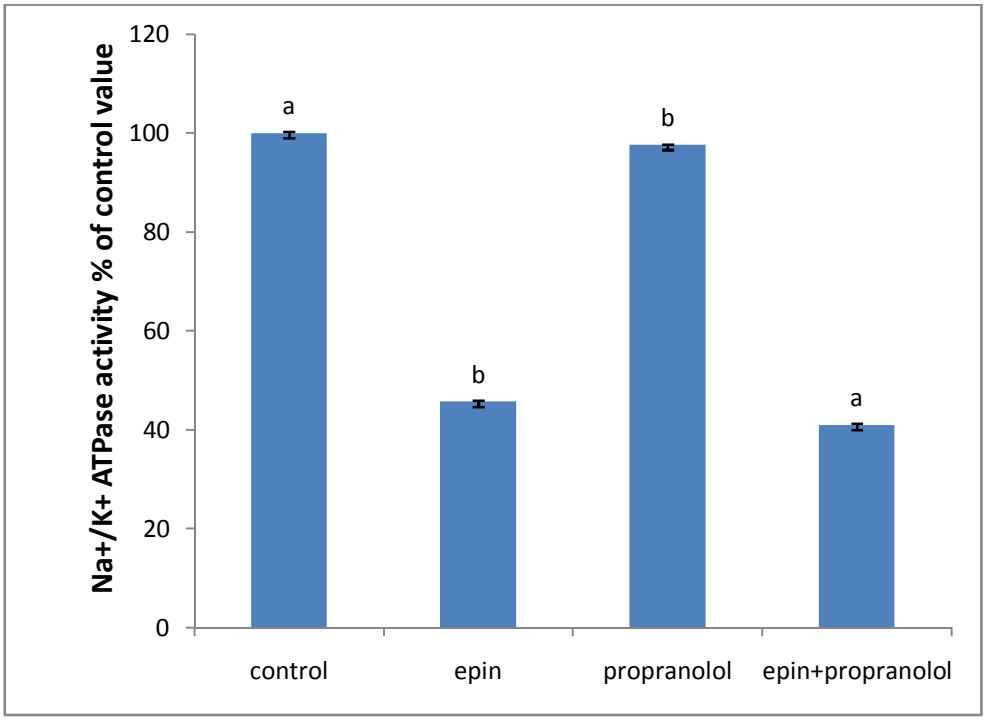

Figure 2a: The effect of epinephrine,propranolol ,and propranolol+epinephrine on the activity of the Na^+/K^+-ATPase. Values are means±SEM .N=3. Bars not sharing a common superscript are considered significantly different from each other at $P<0.05$

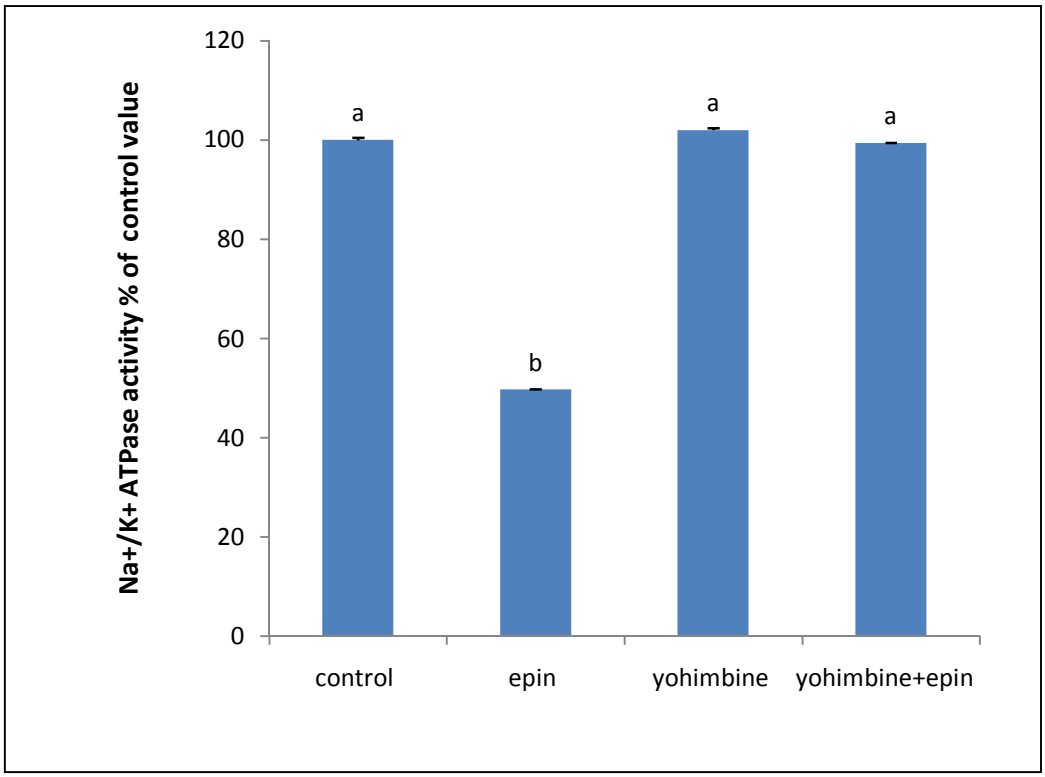

Figure 2b: The effect of yohimbine on the inhibitory effect of epinephrine on the activity of the Na^+/K^+-ATPase. Values are means ± SEM. N=3. Bars not sharing a common superscript are considered significantly different from each other at $P<0.05$.

It is well known that α2-adrenergic receptors are coupled to Gi which acts to down-regulate cAMP and inhibit PKA. Treating the cells with RpcAMP (30μM), a cell permeable PKA inhibitor alone mimicked the inhibitory effect of epinephrine on the Na^+/K^+-ATPase, and didn't result in any additive inhibition when added simultaneously with epinephrine (fig3)

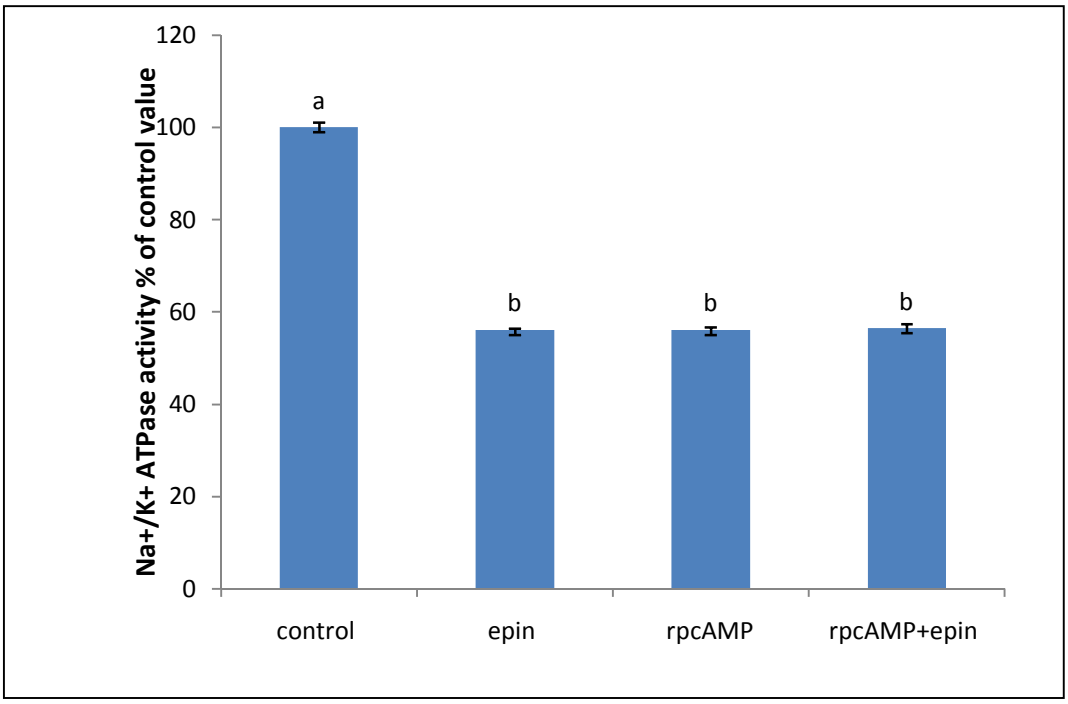

Figure 3: Effect of epinephrine and RpcAMP on the activity of the Na^+/K^+-ATPase. Values are means±SEM. N=3. Bars not sharing a common superscript are considered significantly different from each other at $P<0.01$

C. Determination of the mediators involved

To test the possibility that epinephrine might be signaling through PGE2, NO, PKC, and MEK/ERK, CaCo-2 cells were treated with epinephrine in the presence of their respective inhibitors: Indomethacin, PTIO, CalphostinC, and PD 98059.

The addition of the inhibitors abolished the effect of epinephrine and restored the activity of the Na^+/K^+-ATPase back to control levels (fig4, 5, 6, 7).

The treatment with any of the inhibitors alone didn't cause any significant change in the activity of the Na^+/K^+-ATPase (fig 4, 5, 6, 7).

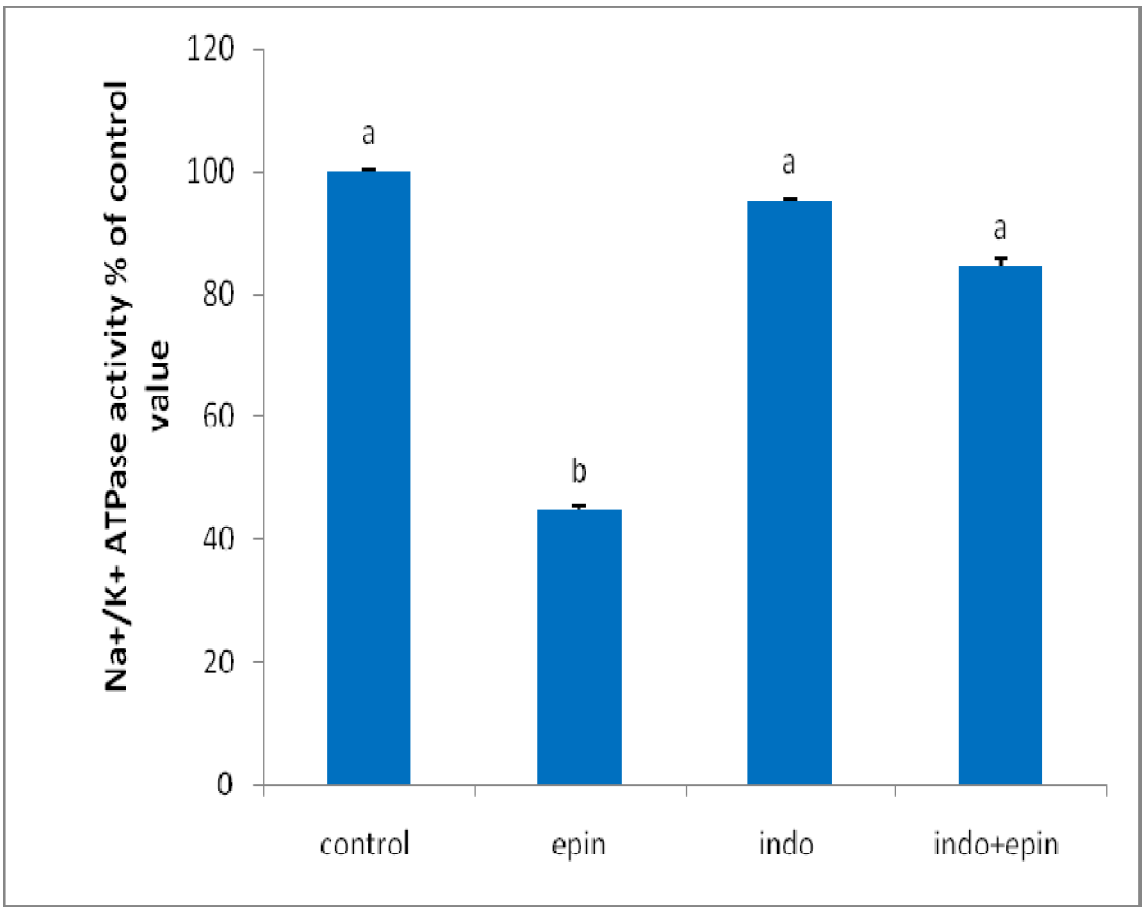

Figure 4: Effect on epinephrine in presence of indomethacin, on the activity of the Na^+/K^+-ATPase .Values are means±SEM. N=5. Bars not sharing a common superscript and are considered significantly different from each other at $P<0.05$.

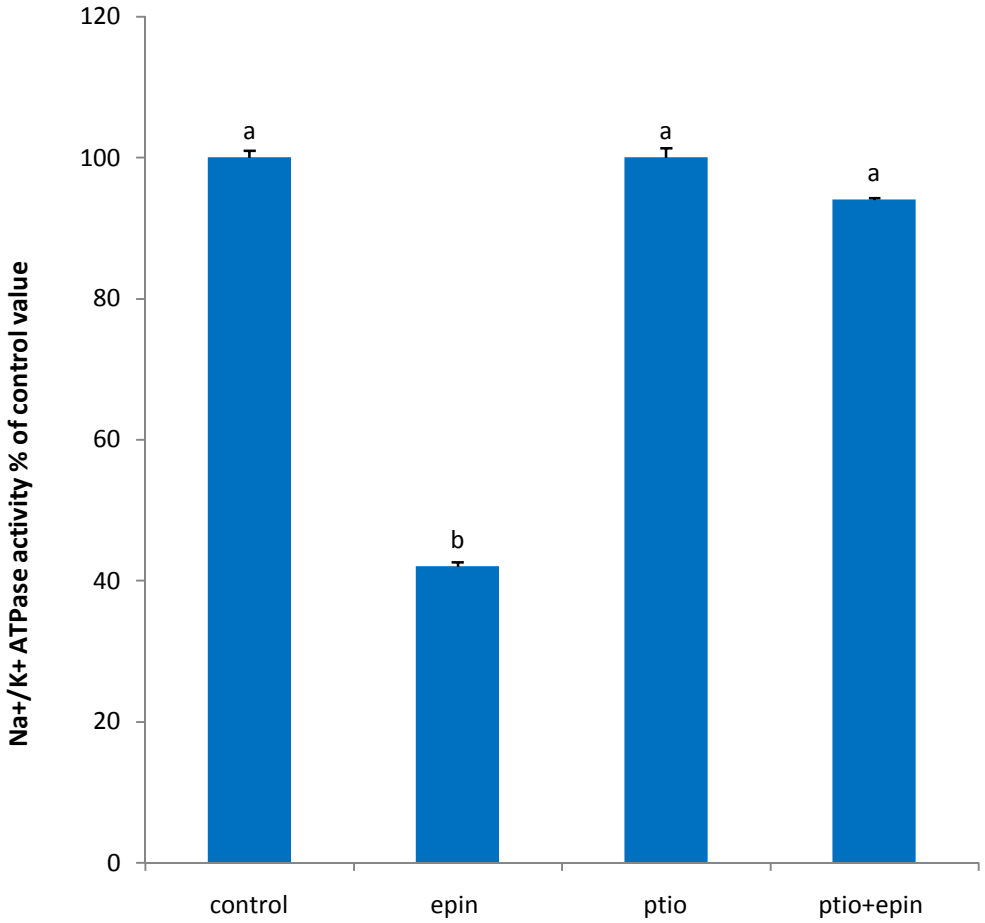

Figure 5: Effect on epinephrine in presence of PTIO, on the activity of the Na^+/K^+-ATPase .Values are means±SEM. N=5. Bars not sharing a common superscript are considered significantly different from each other at P<0.01

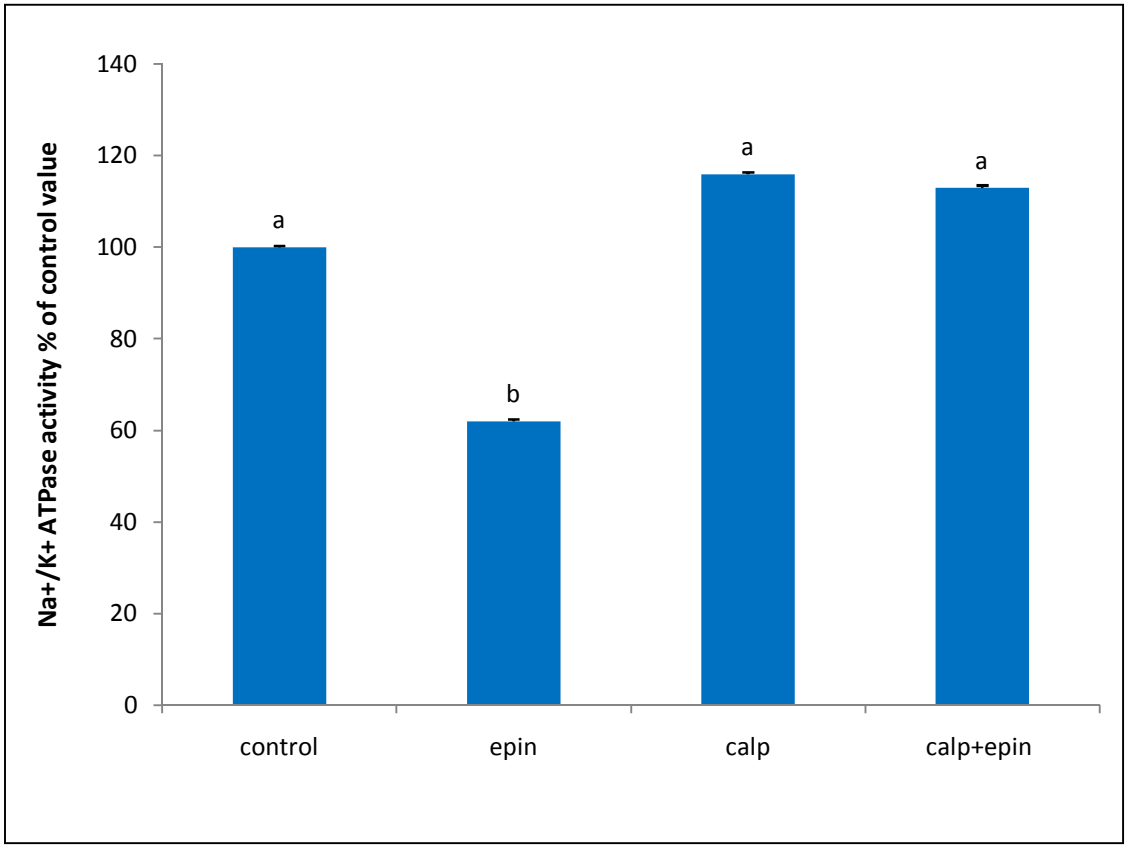

Figure 6: Effect on epinephrine in presence of calphostinC, on the activity of the Na^+/K^+-ATPase .Values are means±SEM. N=6. Bars not sharing a common superscript are considered significantly different from each other at $P<0.05$.

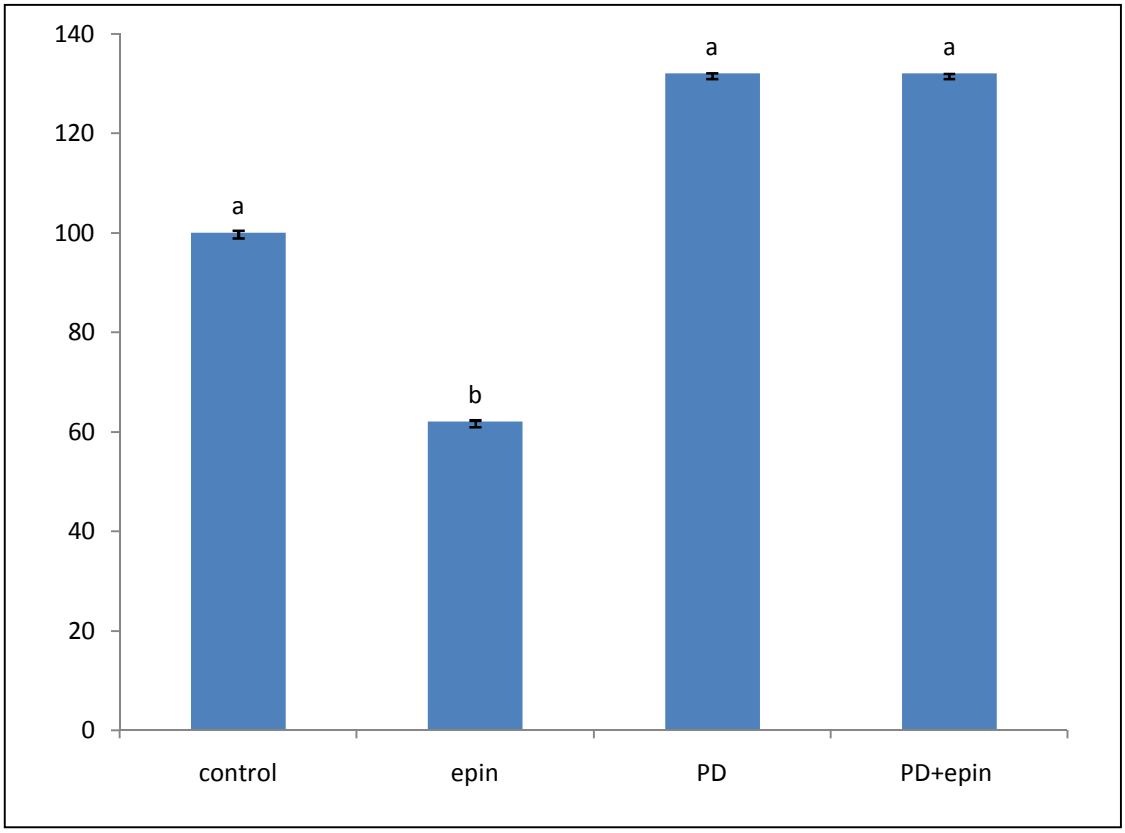

Figure 7: Effect on epinephrine in presence of PD98059 on the activity of the Na^+/K^+-ATPase .Values are means±SEM. N=3. Bars not sharing a common superscript are considered significantly different from each other at P<0.01.

To confirm the involvement of PGE2 in the effect of epinephrine on the pump, the cells were treated with exogenous PGE2 (1nM). The prostaglandin mimicked the effect of epinephrine and reduced significantly the activity of the ATPase (fig 8). A similar inhibitory effect to that of epinephrine was also observed when the cells were treated with SNAP1 , a nitric oxide generator or PMA, a PKC activator (fig9,10).

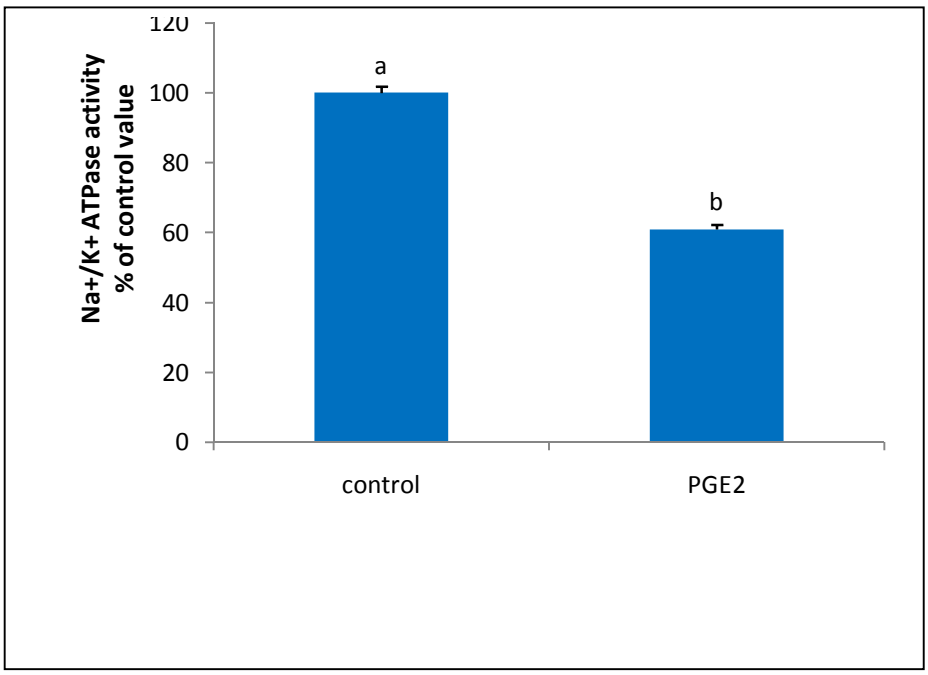

Figure 8: Effect of PGE2 on the activity of the Na^+/K^+-ATPase .Values are means ± SEM. N=6. Bars not sharing a common superscript are considered significantly different from each other at $P<0.05$.

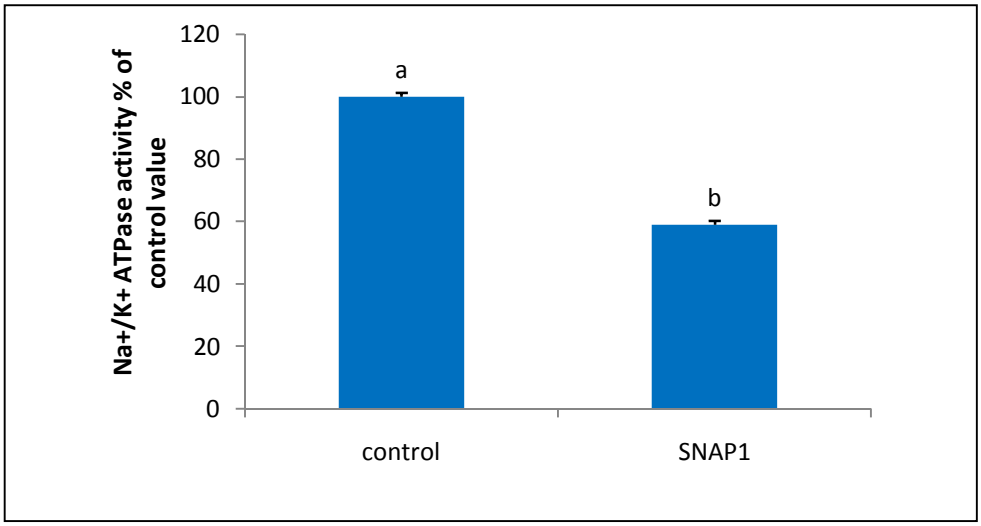

Figure 9: Effect of SNAP-1 on the activity of the Na^+/K^+-ATPase .Values Are means ± SEM. N=5. Bars not sharing a common superscript are considered significantly different from each other at $P<0.05$.

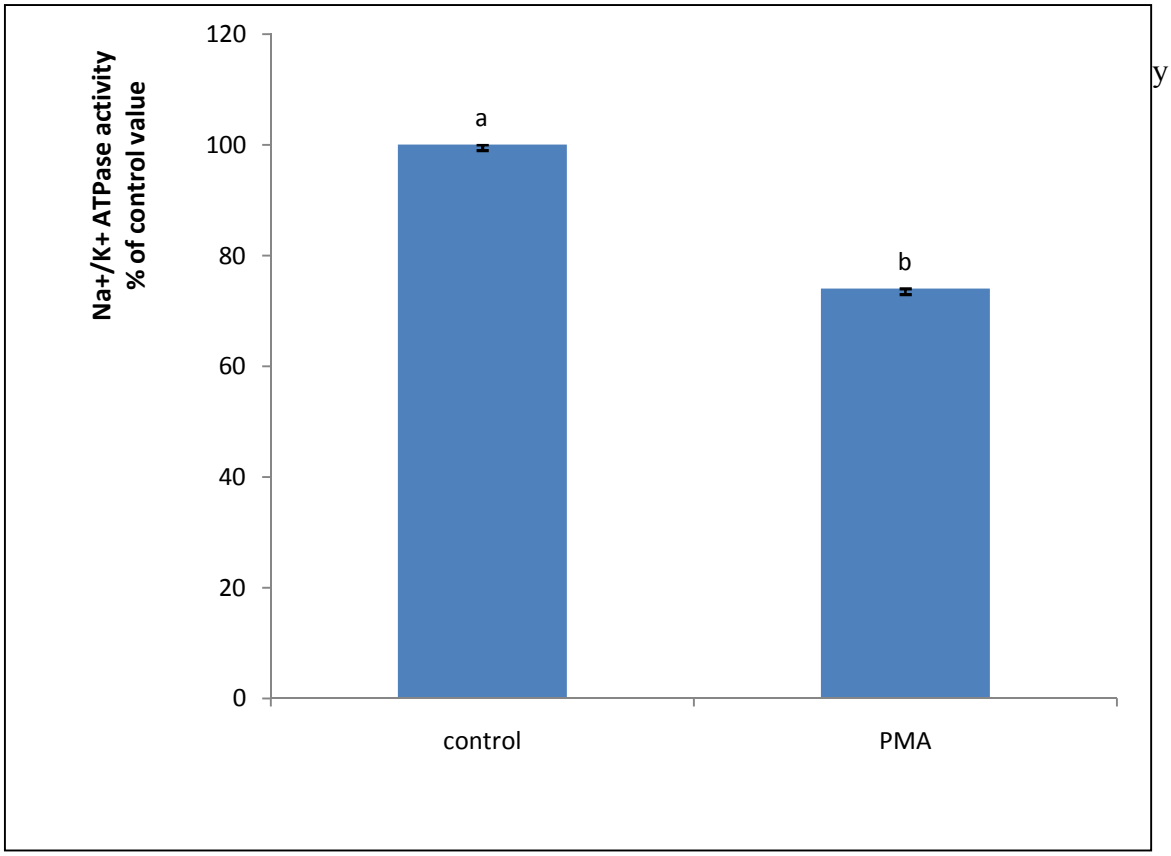

Figure 10: Effect of PMA on the activity of the Na^+/K^+-ATPase .Values are means ± SEM. N=4. Bars not sharing a common superscript are considered significantly different from each other at $P<0.05$.

Taken together, these findings confirm that PGE2, NO, PKC, and MEK /ERK are mediators in the pathway through which epinephrine inhibits the Na^+/K^+-ATPase. The involvement of PKC, led us to suspect that PGE2 acts by binding to its EP1 receptor. This hypothesis was confirmed when the effect of epinephrine completely disappeared in

the presence of SC19220, a selective EP1 antagonist. The antagonist alone had no effect on the activity of the Na^+/K^+-ATPase. (Fig 11).

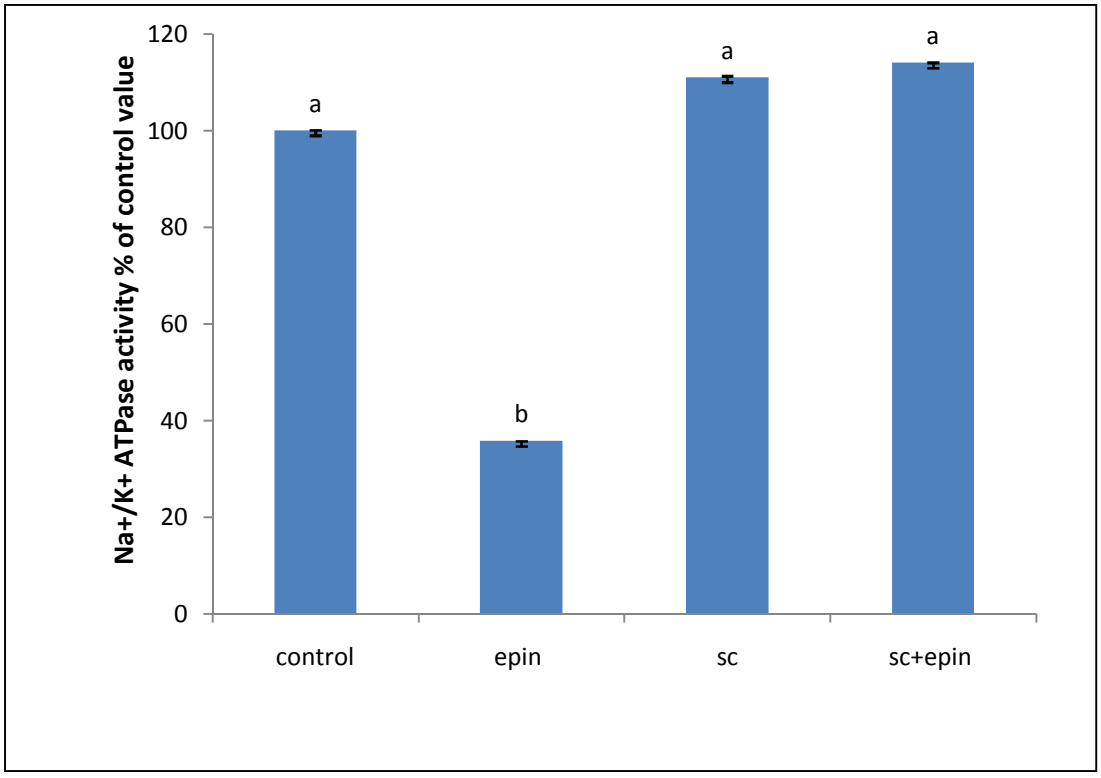

Figure 11: Effect on epinephrine in presence of SC19220 on the activity of the Na^+/K^+-ATPase .Values are means±SEM. N=4. Bars not sharing a common superscript are considered significantly different from each other at $P<0.05$.

D. Positioning the mediators with respect to each other in the pathway

Inhibiting PKC with calphostin abolished completely the effect of PGE2 on the pump. Similarly, in presence of PTIO, a nitric oxide scavenger, the inhibitory effect of PGE2was not manifested (fig12).

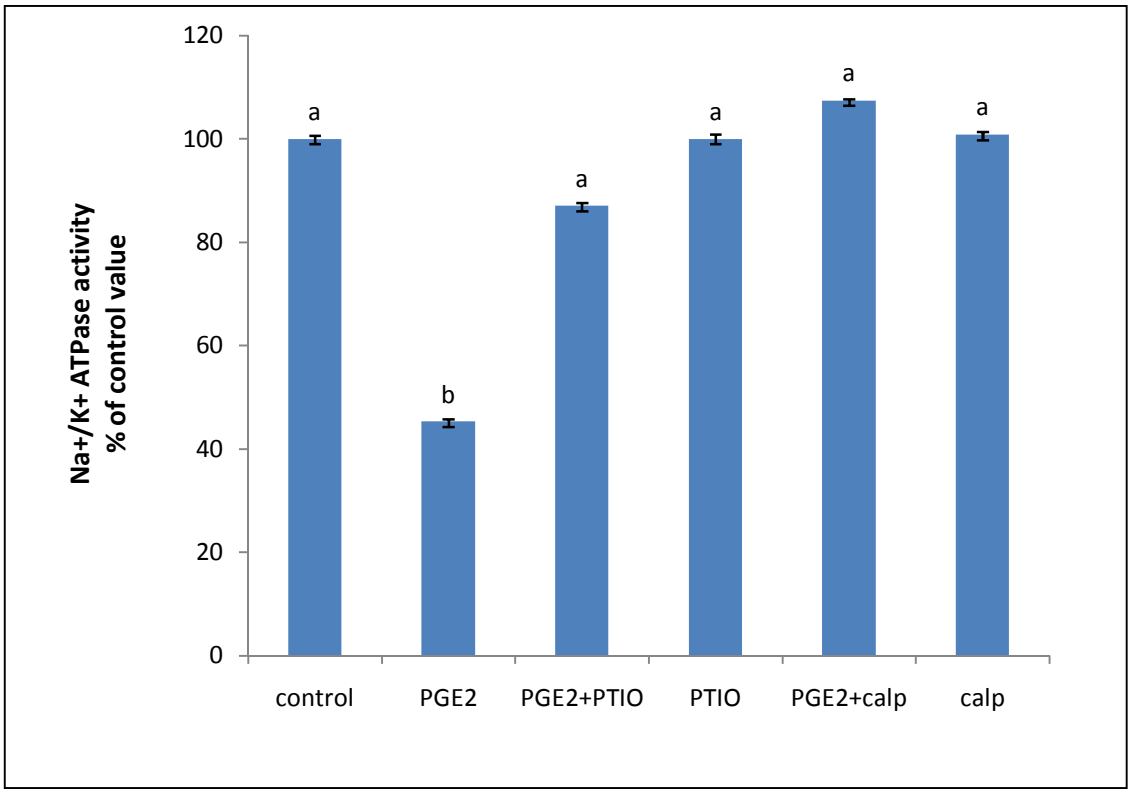

Figure 12: Effect of PTIO and calphostin on PGE2 action on the pump. Values are means±SEM. N=3. Bars not sharing a common superscript are considered significantly different from each other at $P<0.01$.

The effect of PMA on the ATPase disappeared also in presence of PTIO, but persisted in presence of indomethacin. (fig13)

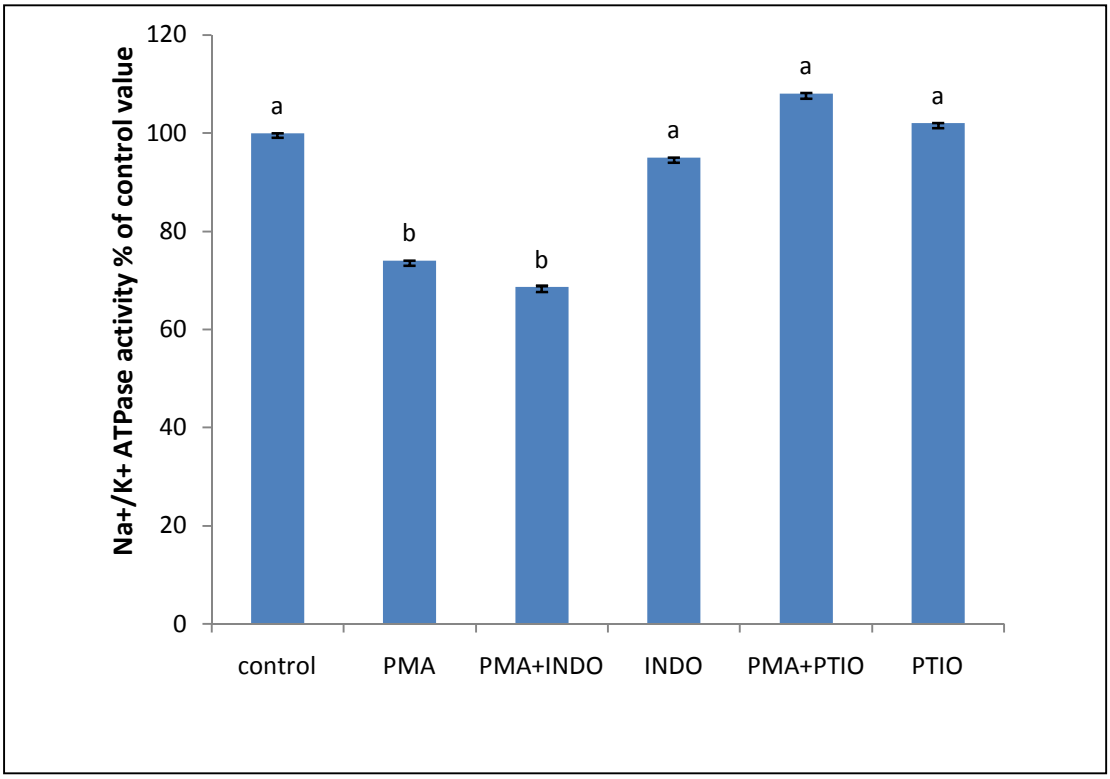

Figure 13: Effect of PMA in presence of ,indomethacin or PTIO , on the activity of the Na^+/K^+-ATPase .Values are means±SEM. N=3. Bars not sharing a common superscript are considered significantly different from each other at $P< 0.01$

CHAPTER V

DISCUSSION

While norepinephrine has been established as a regulator of the Na^+/K^+-ATPase in different cell lines (Adam-Vizi *et al.,* 1979; Ohtomo *et al.,* 1994; Perez-Vizcaino *et al.,* 1999), very few studies so far reported such a role for its derivative epinephrine. Epinephrine was shown to stimulate the Na^+/K^+-pump in rat jejunal crypt cells (Kreydiyyeh, 2000) and skeletal muscles (James *et al.,* 1999), but its mechanism of action was not fully defined. Our work is the first to demonstrate a significant inhibitory effect of epinephrine on the Na^+/K^+-ATPase in Caco-2 cells (Fig1) and to elucidate the signaling pathway involved.

Treatment of CaCo-2 cells with epinephrine for 20 minutes resulted in almost a 50% decrease in the activity of the Na^+/K^+-ATPase.

This negative effect of epinephrine appears to be mediated by α2-adrenergic receptors since it only disappeared in the presence of yohimbine, the specific α2 adrenergic blocker (Fig2a), but persisted when epinephrine was simultaneously added with other adrenergic antagonists (Fig2b). Epinephrine's preferential binding to α2-adrenergic receptors can be attributed to α2 –receptors' higher cell surface density, higher affinity to epinephrine, or both, when compared to other types of adrenergic receptors. The differential characteristics of adrenergic receptors in CaCo-2 cells haven't been addressed before;

nonetheless, α2- receptors, individually, were shown to be highly expressed in a similar adenocarcinoma cell line, HT-29, and to have the highest affinity to epinephrine among other adrenergic agonists (Bouscarel *et al.,* 1984).

In accordance with the widely accepted notion that α2-adrenergic receptors are coupled to inhibitory G-proteins (Gi) and act to down-regulate the production of cAMP and consequently PKA (Taussig *et al.,* 1993), RpcAMP treated cells exhibited the same decrease in Na^+/K^+-ATPase as those treated with epinephrine, and the simultaneous addition of epinephrine and RpcAMP didn't result in an additive inhibition (Fig 3), indicating that epinephrine exerts its effects by inhibiting PKA.

PGE2, NO, PKC, and ERK are familiar regulators of the Na^+/K^+-ATPase. PGE2 and NO were shown to decrease the activity of Na^+/K^+-pump in several tissues including heart (Skayian & Kreydiyyeh, 2006), liver (Kreydiyyeh *et al.,* 2007; Seven *et al.,* 2005), and kidneys (Ominato *et al.,* 1996; Cimen *et al.,* 2004). PKC, via direct or indirect phosphorylation, was observed to lower the affinity of the Na^+/K^+-ATPase to its substrates and to affect the translocation of its subunits to the plasma membrane (Feraille *et al.,* 2000; Dada & Sznajder,1999).Moreover, ERK was reported to contribute to short and long term regulation of Na^+/K^+-ATPase in smooth muscle cells (Isenovic *et al.,*2004), alveolar cells (Zhong *et al.,* 2004), and renal tubule cells (Upadhyay *et al.,*2003).

All four mediators are also known to act downstream of α2-adrenergic receptors. PGE2 was identified as a second messenger for α2-receptor-dependent urea transport and hyperthermic response in rat IMCD (Rouch&Kudo,2000) and guinea pigs (Feleder *et*

*al.,*2004) respectively. α 2-adrenergic stimulation increased NO synthesis in renal circulation (Zou&Cowely,2000),intestinal smooth muscles (Kreiss *et al.,*2004), and thick ascending limb (Plato&Garvin,2000) , and induced ERK phosphorylation in PC12 cells (Karkoulias *et al.,*2005),astrocytes (Peng *et al.,*2003), and proximal tubule cells (Cussac *et al.,*2001).In addition, PKC coupling to α2-adrenergic activation was detected during platelet aggregation(Siess&Lapetina,1989) and vascular smooth muscle contractions (Jinsi-Parimo&Deth,2000).

We therefore hypothesized that the inhibitory effect of epinephrine on the Na^+/K^+-ATPase is mediated by PGE2,NO,PKC, and ERK..The incubation of cells with epinephrine in the presence of inhibitors of these four mediators completely abrogated its effect (Fig4, 5, 6, 7). In addition, treatment with PGE2, SNAP-1, or PMA alone mimicked the inhibitory effect of epinephrine on the Na^+/K^+-ATPase (fig 8,9, 10). Collectively, these results confirmed the involvement of PGE2, NO, PKC, and ERK in the pathway induced by epinephrine.

Next we attempted to locate the mediators with respect to each other in the pathway.PGE2 was found to act upstream of PKC and NO since its negative effect was no longer apparent when it was added in the presence of Calphostin C and PTIO respectively(Fig 12). The effect of PMA, however, only disappeared when added with PTIO, but persisted in the presence of indomethacin (Fig13). These results indicate that PKC acts upstream of NO and further validate its position downstream of PGE2.

The obtained order of the mediators (PGE2→PKC→NO) is in line with previous reports in the literature. Hori *et al* (2000) and Uno *et al.,* (2004) demonstrated that PGE2 enhances the release of NO via the activation of NOS in rat intestinal macrophages and gastric mucosa respectively. More importantly, the PGE2 induced increase in NOS activity and NO/cGMP production in rat submandibular gland was found to be mediated by PLC and PKC (Borda *et al.,* 2002). A tissue-specific role of PKC in the regulation of the different NOS isoforms was reported in several studies and was attributed to either direct phosphorylation at certain residues (Vasilets,1997& Efendiev *et al.,*2000) or indirect phosphorylation via the activation of the mitogen-activated protein kinase cascade(Wen *et al.,*2011).

Pharmacological studies have revealed that PGE2 is capable of binding to four subtypes of G-protein coupled E-prostanoid receptors (EP1-4) (Bose *et al.,* 2004). Upon the stimulation of EP1 Gq-coupled receptor, Gαq is released to activate PLC –dependent PIP2 hydrolysis and increase intracellular Ca2+ concentrations and PKC activity via IP3 and DAG respectively (Herbert *et al.,*1990).The fact that PGE2 was found to induce the activation of PKC in the pathway hinted that PGE2 is signaling by binding to its EP1 receptor. Indeed, the incubation of the cells with SC 19220 (selective blocker of EP1) , prior to the treatment of epinephrine, completely abolished the inhibitory effect of epinephrine on the Na^+/K^+-ATPase (Fig11), thus verifying that PGE2 mediates the effect of epinephrine by selectively binding to EP1 receptor.

NO was found to be the most downstream mediator in the pathway; nonetheless, its mechanism in the regulation of the Na^+/K^+-ATPase remains to be determined. Two modes of NO-related Na^+/K^+-ATPase regulation have been described: cGMP-dependent and cGMP-independent regulation.

Soluble guanylyl cyclase (sGC) is the most well recognized physiological receptor for NO (Lucas *et al.,* 2000). The binding of NO to the heme group of sGC will induce its ability to synthesize cGMP, a potent activator of PKG (Martin *et al.,* 2005). cGMP/PKG was reported to reduce the activity of the Na^+/K^+-ATPase in various cell lines including, mouse proximal tubule epithelial cells (Guzman *et al.,*1995), opossum kidney cells (Liang & Knox,1999),renal medulla (Beltowski *et al.,*2003), and non-pigmented ciliary epithelium of porcine eye (Shahidullah & Delamere, 2006). Whether PKG however, alters the activity of the Na^+/K^+-ATPase directly via α-subunit phosphorylation or indirectly by the phosphorylation of other regulatory proteins is still under investigation.

Structural analysis revealed that PKA and PKG share common phosphorylation consensus sequences in substrate proteins (Wood *et al.,* 1996). Since PKA is known to directly target the Na^+/K^+-ATPase (Feschenko*et al.,* 1995), PKG, therefore, should be capable of interacting with and phosphorylating the Na^+/K^+-ATPase; nonetheless, to our knowledge , very few studies so far supported such a function whereby PKG was reported to phosphorylate the α- 1 subunit of Na^+/K^+-ATPase purified from the dog, sheep , pig, rat kidney , and Xenopus Oocyte at unidentified residues (Fotis *et al.,*1999). This

phosphorylation, however, resulted in the stimulation rather than the inhibition of the Na^+/K^+-ATPase.

PKG-indirect regulation of the Na^+/K^+-ATPase, on the other hand, is better understood and was shown to involve a variety of signaling mediators. cGMP/PKG elicited the phosphorylation and activation of DARPP-32 and protein phosphatase inhibitor -1(I-1) to inhibit protein phosphatase-1 and reduce the activity of the Na^+/K^+-ATPase in tubular (Meister *et al.,*1989) and kidney cells (Li *et al.,*1995). Furthermore, NO-dependent decrease in aqueous humor secretion in porcine eye was shown to be a consequence of cGMP/PKG- mediated ERK1/2 and p38-MAPK induction, and subsequent Na^+/K^+-ATPase inhibition following its phosphorylation at Tyr-10 (Shahidullah *et al.,* 2014). Other cGMP-dependent mechanisms in the modulation of the Na^+/K^+-ATPase include the regulation of phosphodiesterases such PDE2 and PDE3.cGMP was shown to stimulate PDE 2 and inhibit PDE3 to down-regulate and up-regulate intracellular cAMP respectively (Beltowski *et al.,*2003). NO inhibited Na^+/K^+-ATPase in leptin-treated renal cells by the subsequent stimulation of cGMP, activation of PDE2 , and decrease in cAMP concentrations (Beltowski *et al.,*2007). Likewise,an increase in cAMP was also reported to negatively affect the Na^+/K^+-ATPase, as observed in rat renal cortex (Berterello *et al.,*1991& Fisoni *et al.,*1994) and mice hippocampus neurons (Wu *et al.,*2006) , but via a mechanism independent of PDE3 ; nonetheless, the possibility that PDE3 can be responsible for such effects in other tissues can't be ruled out.

A far less common mode of NO-mediated Na^+/K^+-ATPase regulation is through cGMP-independent mechanisms. By interacting with other endogenously produced anions, such as superoxide, NO has the potential to generate free radical compounds that were shown to cause lipid oxidation and disrupt protein functions mainly by nitrosylating or nitrating critical amino acid residues (Beckman & Crow, 1993 & Lipton *et al.*, 1993). Guzman *et al.* reported a role for peroxynitrite in the NO-induced inhibition of Na^+/K^+-ATPase in mouse proximal tubule epithelial cells (1995). Moreover, nitrosylation of Na^+/K^+-ATPae cysteine residues and thiol groups significantly reduced its activity in NO-treated brain and kidney tissues (Boldyrev *et al.,* 1997). Other modification may include phosphorylation by NO-activated PKC as observed in OK cells that exhibited a PKG-independent but PKC-dependent decrease in Na^+/K^+-pump activity upon incubation with the NO generator SNP (Liang & Knox, 1999).

In conclusion, our work demonstrated for the first time the effect of epinephrine on the Na^+/K^+-ATPase in CaCo-2 cells, and described its mechanism of action by elucidating the underlying signaling pathway.

The colon, or the large intestine, constitutes the major site for water and electrolyte absorption. The rate of colonic water absorption is directly dependent on the rate of Na+ influx, whereby the uptake of Na^+ by the cells will cause water to follow by osmosis (Sandle GI., 1998). The Na^+/K^+-ATPase is responsible for maintaining a low intracellular concentration of Na^+ ions in colon cells, thus providing a driving force for the passive entry of Na^+ from the lumen into the cytosol (Kunzelmann & Mall, 2002). A decrease in

the activity of the Na^+/K^+-ATPase is very likely to result in a decrease in colonic water absorption. Whether epinephrine, via the described pathway, could affect water movement in the colon is the aim of future investigations.

Figure 14 below summarizes the signaling pathway of epinephrine on the Na^+/K^+-ATPase

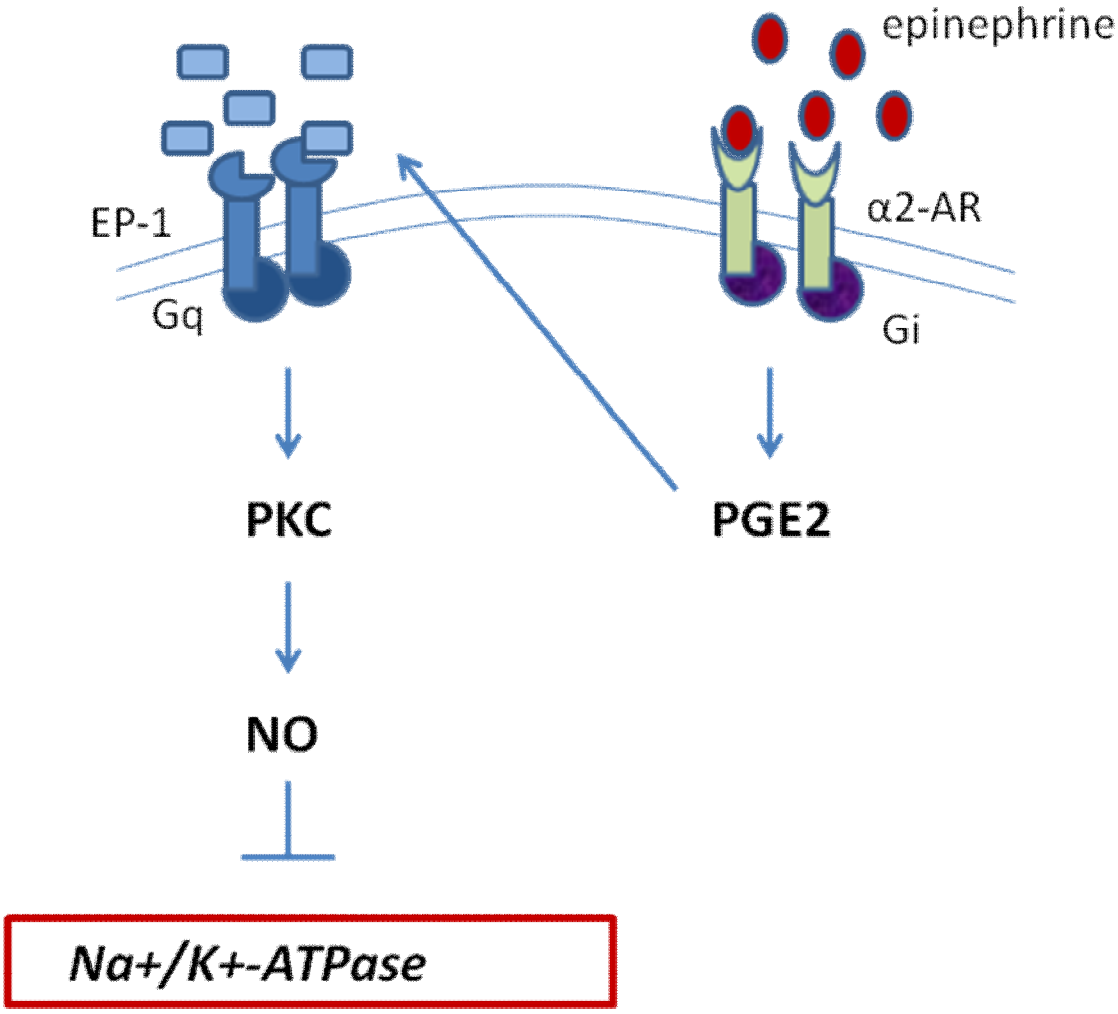

Figure14: Proposed signaling pathway for the action of epinephrine on the Na^+/K^+-ATPase .Arrows with pointed ends: stimulatory effect; arrows with blunt end: inhibitory effect.

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