AMERICAN UNIVERSITY OF BEIRUT

FTY720P STIMULATES THE Na^+/K^+ ATPASE IN CACO-2 CELLS VIA PKC, PGE2 AND PKA

by
MAYSOON ISSAM NOUREDDINE

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MAYSOON ISSAM NOUREDDINE

Approved by:

Dr. Sawsan Kreydiyyeh, Professor — Advisor
Biology

Dr. Khouzama Knio, Professor — Member of Committee
Biology

Dr. Zakaria Kambris, Associate Professor — Member of Committee
Biology

Date of thesis defense: [April 27, 2018]

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AN ABSTRACT OF THE THESIS OF

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Title: FTY720P Stimulates the Na^+/K^+ ATPase in Caco-2 Cells via PKC, PGE2, and PKA

Na^+/K^+ ATPase is ubiquitously expressed in all animal cells serving a myriad of fundamental functions. In the intestines, it regulates sodium, water, and nutrient absorption; hence, any significant alteration in its activity can trigger the onset of diarrhea or constipation. Patients with Inflammatory Bowel Disease (IBD) suffer from diarrhea with their colonic mucosal tissues showing elevated sphingosine-1-phosphate (S1P) levels. Previous studies in our lab have shown a modulatory effect of S1P on the activity of Na^+/K^+ ATPase in HepG2 cells. This work was undertaken to examine whether S1P exerts a similar modulatory effect in colonocytes, and in case it does, to determine the signaling pathway. FTY720P was used as an analogue of S1P and Caco-2 cells as a model. Na^+/K^+ ATPase activity was assayed by measuring the amount of inorganic phosphate liberated in the presence and absence of ouabain, a specific inhibitor of the ATPase. A time-response study, conducted between 75 and 180 min, revealed a significant stimulatory effect of FTY720P on Na^+/K^+ ATPase, reaching maximal effects at 90 min and beyond. Two hours were considered as the optimal period for FTY720P action and were adopted in all subsequent experiments. The stimulatory effect of FTY720P was completely abolished in presence of JTE-013, a S1P receptor 2 (S1PR2) antagonist but not in the presence of CAY10444, a blocker of S1P receptor 3 (S1PR3). Furthermore, the S1PR2 agonist, CYM5520, stimulated the Na^+/K^+ ATPase in a similar fashion to that of FTY720P while CYM5541, a S1PR3 agonist, had no effect. The effect of FTY720P completely disappeared also in presence of calphostin (PKC inhibitor), BAPT/AM (calcium chelator), NFκB inhibitor, indomethacin (COX inhibitor), RpcAMP (PKA inhibitor), and Wortmannin (PI3K inhibitor) and was mimicked by PMA (PKC activator), exogenous PGE2 (prostaglandin E2), and dbcAMP (PKA activator). The stimulatory effect of PMA disappeared completely when Caco-2 cells were pre-treated with NFκB inhibitor, Indomethacin, RpcAMP, or wortmannin. On the other hand, only RpcAMP and wortmannin completely suppressed PGE2-induced Na^+/K^+ ATPase activation. Upon blocking EP3 receptor with L798106, FTY720P was still capable of activating the Na^+/K^+ ATPase. In addition, sulprostone (EP1 and EP3 receptors agonist) and Butaprost (EP2 receptor agonist) did not cause any change in the activity of the ATPase. Western blot analysis showed that dbcAMP, as well as PGE2 and wortmannin, inhibit ERK1/2. A more prominent reduction in ERK1/2 activity was observed in cells treated with PGE2 in

presence of wortmannin. However, inhibiting ERK1/2 with PD98059 did not affect the FTY720P-induced Na^+/K^+ ATPase stimulation, indicating that although the activity of ERK1/2 is modulated by PKA, PI3K and PGE2, yet the kinase is not along the pathway leading to the ATPase activation. It was concluded that in Caco-2 cells, FTY720P activates the Na+/K+ ATPase when applied for 2 hours by binding to S1P receptor 2 leading to the sequential activation of conventional PKC and NF-κB. The transcription factor then induces PGE2 synthesis. PGE2, in turn, binds to its EP4 receptor and activates PKA and PI3K, inducing Na^+/K^+ ATPase stimulation.

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ABBREVIATIONS

3,5-T_2	3,5-diiodothyronine
AC	Adenylyl cyclase
AKAPs	A-kinase anchoring proteins
ATP	Adenosine 5’ triphosphate
Akt	Protein kinase B
BSA	Bovine serum albumin
CAC	Colitis associated colorectal cancer
cAMP	Cyclic adenosine monophosphate
CCV	Clathrin-coated vesicles
CD	Crohn’s disease
COX	Cyclooxygenase
D1	Dopamine 1
D2L	Dopamine 2 long receptor
DAG	Diacylglycerol
DARPP-32	Dopamine and cAMP-regulated neuronal phosphoprotein Mr-32,000
dbcAMP	Dibutyryl adenosine 3’ – 5’ - monophosphate
DMEM	Dubilico’s minimal essential medium
EDG	Endothelial differential gene
EGF	Epidermal growth factor
ERK1/2	Extracellular signal regulated kinase

FBS	Fetal bovine serum
FTY720	Fingolimod
FTY720P	Fingolimod phosphate
GI	Gastrointestinal
GPCR	G-protein coupled receptor
GRK-2	G-protein coupled receptor kinase 2
HRP	Horse radish peroxidase
IBD	Inflammatory bowel disease
IgG	Immunoglobulin G
IL-1β	Interleukin-1 β
IL-6	Interleukin-6
IκB	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor
IP_3	1,4,5- inositol triphosphate
LPP3	Lysophospholipid phosphatase 3
MAPK	Mitogen-activated protein kinase
MS	Multiple Sclerosis
NADPH	Nicotinamide adenine dinucleotide phosphate
NF-κB	Nuclear factor kappa light chain enhancer of activated B-cells
NHLF	Normal human lung fibroblasts
NO	Nitric oxide
PKA	Protein kinase A
PKC	Protein kinase C

PDE	Phosphodiesterase
PGE2	Prostaglandin E2
PI3K	Phosphoinositide-3-kinase
PIP-2	Phosphatidylinositol 4,5-bisphosphate
PLA-2	Phospholipase A2
PLC	Phospholipase C
PMA	Phorbol-12-myristate-13-acetate
PP-1	Protein phosphatase 1
PTH	Parathyroid hormone
ROS	Reactive oxygen species
RpcAMP	Monophosphorothioate, Rp-isomer Triethyl ammonium salt
S1P	Sphingosine-1-phosphate
S1PR	Sphingosine-1-phosphate receptor
SphK	Sphingosine kinase
T_3	Triiodothyronine
TGF-β	Transforming growth factor-β
TNF-α	Tumor necrosis factor-α
UC	Ulcerative Colitis

CHAPTER I

INTRODUCTION

Diarrhea is a prevalent disorder affecting people worldwide. Around 1.5 billion cases are reported every year with approximately 1.5 million episodes leading to death. Causes of diarrhea include aberrant mechanisms of osmosis, secretion, motility, and inflammation at the level of the gastrointestinal tract (GI-tract) (Dinesen & Harbord, 2013).

Diarrhea is one of the symptoms of inflammatory bowel disease (IBD), a chronic condition that causes mucosal inflammation in the GI-tract. It encompasses 2 diseases: ulcerative colitis (UC) and Crohn's disease (CD). IBD patients suffer from fever and stomachache and are more prone than healthy people to GI-tract puncturing, infections, and colon cancer. Moreover, children and adolescents having IBD may experience a halt in their development (Suh & Saba, 2015).

In IBD, a lower activity of Na^+/K^+ ATPase was observed which would reduce the sodium gradient across the apical membrane of intestinal cells, and consequently the absorption of sodium and water (Greig & Sandle, 2006). IBD is accompanied also with higher levels of sphingosine-1-phosphate (S1P), attributed to a higher expression of sphingosine kinase (SphK), and S1P receptors in colonocytes (Suh & Saba, 2015). These observations suggest a possible relationship between S1P and Na^+/K^+ ATPase. Fingolimod phosphate (FTY720P) is a S1P analogue, used in the treatment of Multiple Sclerosis (MS). MS is an autoimmune disease characterized by the egression of mature lymphocytes from the lymph nodes into the blood and their establishment in the central nervous system where they induce neurodegeneration (Chun & Hartung, 2010).

FTY720P was found to prevent the egression of lymphocytes from the lymph nodes and to repair and support damaged neurons in the brain. It works thus, at both the immunological and neural levels (Brinkmann, 2009). Whether this drug works also at the level of colonic transport processes is a question that has not been addressed before. The colon absorbs electrolytes and water and compacts the feces in preparation for their elimination. The main electrolyte absorbed is sodium, and the process is geared by the sodium gradient established by the Na^+/K^+ ATPase. Consequently, any change in the activity of the ATPase is expected to alter sodium and water absorption and lead to diarrhea or constipation.

The aim of this work was to investigate the role of FTY720P, as a S1P analogue, in modulating the activity of colonic Na^+/K^+ ATPase and to determine the signaling molecules involved. The results will not only uncover the side effects of FTY720P on colonic activities but will also reveal the mechanism of action of the drug and its signaling pathway, providing thus a possibility of circumventing any undesirable effect by targeting the specific intermediates involved.

CHAPTER II

LITERATURE REVIEW

A. Sodium-Potassium ATPase

The Na^+/K^+ ATPase is expressed ubiquitously in the plasma membrane of all animal cells (Glynn, 1985). It pumps 3 sodium ions to the outside of the cell and 2 potassium ions to the inside for every ATP hydrolyzed (Blanco & Mercer, 1998).

1. Structure

Na^+/K^+ ATPase is a P-type ATPase and thus it interconverts between two distinct conformations: E1 and E2. The E1 is the conformation having the Na^+ and K^+ binding sites directed towards cytoplasm while the E2 is the conformation having the latter binding sites facing the extracellular matrix (Horisberger, Lemas, Kraehenbühl, & Rossier, 1991). Structurally, a functional Na^+/K^+ ATPase is made up of 2 subunits: a catalytic alpha (α) subunit and a glycoprotein beta (β) subunit; an additional accessory FXYD subunit can also be present (Clausen, Hilbers, & Poulsen, 2017).

The α-subunit is comprised of around 1,000 amino-acids and has a molecular weight of 110 kDa. It consists of 10 transmembrane domains with 5 extracellular loops and intracellular N and C termini (Kaplan, 2002). It includes binding sites for sodium, potassium, ATP, phosphate, and ouabain (Horisberger et al., 1991). Mammals express 4 distinct isoforms of the α-subunit: α_1, α_2, α_3, and α_4. Alpha-1 is expressed in almost all tissues and most abundantly in kidney and epithelial cells, while the α_2 isoform is expressed in cardiac and skeletal muscle cells as well as in glial cells and astrocytes. The α_3 isoform is found predominantly in the brain and particularly in neuronal

projections. The α_4 isoform has been identified in the cell membranes of sperm (Clausen et al., 2017). The main function of the catalytic α-subunit is to hydrolyze ATP to generate the needed energy for the pumping of sodium and potassium ions across the plasma membrane against their electro-chemical gradient (Geering, 2008).

The β-subunit consists of approximately 370 amino-acid residues and is made up of 1 transmembrane segment with the C-terminus projecting towards the extracellular matrix and the N-terminus present in the cytoplasm. Four β isoforms (β_1, β_2, β_3, β_4) have been identified having each a molecular mass of around 55 kDa. The β-subunit plays a fundamental role in the maturation of the Na^+/K^+ ATPase. It acts as a chaperone by establishing appropriate folding and integration of the α-subunit into the cell membrane ensuring its proper functioning and its preservation from degradation (Geering, 2001).

As for the FXYD subunit, 7 isoforms- FXYD1, 2, 3, 4, 5, 6, 7- are expressed in mammals. FXYD1 is found abundantly in cardiac and skeletal muscle cells as well as in brain neurons. FXYD 7 and 8 are mostly found in the brain. Both FXYD2 and FXYD4 are predominantly expressed in the kidneys while FXYD3 and FXYD5 are overexpressed in cancerous cells (Clausen et al., 2017). The main role of the FXYD subunit is to regulate the activity of the Na^+/K^+ ATPase by modulating the affinity of the α-subunit to each of the sodium and potassium ions as well as affecting the maximum current under saturated substrate conditions I_{max} (Geering, 2008).

Different associations can form between the α, β, and FXYD isoforms resulting in slightly distinct Na^+/K^+ ATPases that differ in their kinetic properties and affinities (Clausen et al., 2017). Specific expression patterns of the ATPase isozymes occur (Blanco & Mercer, 1998), depending on the developmental stage and the identity of the cells and tissues.

2. Regulation of the Na^+/K^+ ATPase

a. Short-term Regulation of Na^+/K^+ ATPase

Transient changes in Na^+/K^+ ATPase activity may result from a modification in the amount of ions pumped across the plasma membrane per ATPase molecule per unit time or a modification in the affinity of the ATPase to the substrate (Na+ ions). Moreover, a change in the amount of pump molecules translocated from the intracellular stores to the plasma membrane of the cell is another form of short-term alteration of ATPase activity (Ewart & Klip, 1995).

b. Long-term Regulation of Na^+/K^+ ATPase

Long-term changes in the activity of the Na^+/K^+ ATPase result from mechanisms that affect transcription, translation and the stability of transcripts and protein subunits of the pump (Ewart & Klip, 1995).

c. Regulators of Na^+/K^+ ATPase

It has been established that the activity of the ATPase is influenced by intracellular Na^+ and ATP concentrations as well as by the extracellular K^+ concentration. However, the most dramatic effect would be that of intracellular Na^+ concentration since, under physiological conditions and in most tissues, the Na^+/K^+ pump is saturated with ATP and K^+ ions but not with Na^+ ions. Therefore, a slight increase in the intracellular Na^+ ion concentration can significantly augment the activity of the pump (Therien & Blostein, 2000).

Cytoskeletal components play also a fundamental role in the regulation of the Na^+/K^+ ATPase activity. For instance, monomeric actin undergoes polymerization to

produce actin filaments of a particular length which can bind to the pump and induce a conformational change that results in an increase in its affinity to Na^+ and its stimulation. Protein kinase A (PKA) can abolish this stimulatory effect (Cantiello, 1995; Cantiello, 1997). Interaction between the pump and the cytoskeletal components was reported to be a crucial step in its trafficking between intracellular stores and cell membrane (Therien & Blostein, 2000).

Membrane components together with their derivatives and metabolites can impact the activity of Na^+/K^+ ATPase. Free fatty acids and intracellular membrane lipid derivatives such as sphingosine, phosphatidyl choline, and oleic acid, also known to be regulators of Protein kinase C (PKC) (Hannun, Loomis, Merrill, & Bell, 1986; Oishi, Raynor, Charp, & Kuo, 1987; Oishi, Zheng, & Kuo, 1990), have been shown to reduce the pump's activity in CHO-K1 cells. This inhibition was due to an interaction of the latter molecules with the Na^+ binding site on the pump (Oishi et al., 1990).

Na^+/K^+ ATPase activity can also be regulated by cardiac glycosides, such as ouabain. The inhibitory effect of ouabain on the pump is well-established in the literature (Aperia, 2001). A role in promoting endocytosis and degradation of pump molecules (Tian et al., 2009) has also been observed. However, treating cells with minute concentrations of ouabain can initiate a signaling cascade that varies with the cell type and its microdomain constitution. In opossum kidney cells, long-term treatment with ouabain induced an increase in the activity of the ATPase and in the expression of its α_1 subunit by activating the MAPK and PI3K/Akt pathways while, in PY-17 cell line, ouabain had no effect on the expression of the pump (Silva & Soares-Da-Silva, 2011).

Hormones are also important regulators of Na^+/K^+ ATPase. Dopamine, a catecholamine with natriuretic effect, inhibits the Na^+/K^+ ATPase in a variety of cells including arterial (Rashed & Songu-Mize, 1995), lung (Barnard et al., 1997), kidney (Aperia et al., 1991; Satoh, Cohen, & Katz, 1992), and small intestinal cells (Vieira-Coelho, Lucas Teixeira, Finkel, Soares-Da-Silva, & Bertorello, 1998). In OK cells, binding of dopamine to its respective G-protein coupled receptor (GPCR) induces PKC activation. The latter phosphorylates the α-subunit of Na^+/K^+ ATPase at ser-18 residue causing its internalization into clathrin-coated vesicles (CCV) which translocate the phosphorylated ATPase to early and late endosomes (Chibalin et al., 1999). Another mechanism works in tandem with the latter pathway to cause a dopamine-induced inhibition of the pump. It is initiated by the binding of dopamine to its GPCR (G_s) and activation of adenylyl cyclase (AC), resulting in an increase in cAMP levels and PKA activation. PKA phosphorylates dopamine and cAMP regulated phosphoprotein of Mr 32,000 (DARPP-32) which inhibits protein phosphatase 1 (PP-1). Inhibited PP-1 can no longer dephosphorylate Na^+/K^+ ATPase, thus, leading to an increase in the amount of phosphorylated inactive pump. On the other hand, a dopamine 2 long (D2L) receptor agonist can elicit a stimulatory effect on the Na^+/K^+ ATPase in LTK-cells by suppressing cAMP production (Aperia et al., 1991; Fryckstedt, Meistert, & Aperia, 1992).

Adrenergic catecholamines modulate also the activity of Na^+/K^+ ATPase through protein kinases, specifically PKA and PKC, in a tissue-dependent manner (Therien & Blostein, 2000). In fat cells (Horwitz & Eaton, 1975), kidney (Giesen, Imbs, Grima, Schmidt, & Schwartz, 1984), and stomach (Moore & Fay, 1993), catecholamines generally bind to their β-adrenergic receptors and increase the activity

of Na^+/K^+ ATPase by increasing cAMP levels which activate PKA (Therien & Blostein, 2000). However, Fisone et al. (1998) demonstrated that stimulation of the β-adrenergic receptors in the choroid plexus leads to an inhibition of the ATPase. This inconsistency can be explained by the study conducted by Kiroytcheva et al. (1999) which shows that activated PKA can have different effects on the pump depending on whether oxygen is present or not. Under deoxygenated conditions, PKA elicits an inhibitory effect on the pump while under oxygenated conditions, it causes pump activation. On the other hand, in liver cells, adrenergic catecholamines usually bind to their α-adrenergic receptors to induce a stimulatory effect on the pump through PKC activation (Lynch, Wilson, Blackmore, & Exton, 1986). Besides, in renal proximal tubules, activating α-adrenergic receptor by its agonist oxymetazoline leads to an increase in intracellular Ca^{2+} concentration which in turn activates the calcium/calmodulin dependent phosphatase, calcineurin. Calcineurin may either dephosphorylate the Na^+/K^+ ATPase directly leading to its activation or dephosphorylate and inactivate DARPP-32-PO_4 thus preventing it from inhibiting protein phosphatase 1 (PP-1). Active PP-1 can now dephosphorylate the Na^+/K^+ ATPase leading to its activation through this indirect pathway (Aperia, Ibarra, Svensson, Klee, & Greengard, 1992).

Another hormone that can regulate the activity of Na^+/K^+ ATPase is the thyroid hormone. It has been proposed that Triiodothyronine (T_3) and 3,5-diiodothyronine (3, 5-T_2) hormones but not thyroxine induce a cascade of signaling events that lead to the inhibition of Na^+/K^+ ATPase in chick embryo hepatocytes in an age-dependent manner. This cascade involves the activation of each of PKC and PKA through the action of phospholipase C (PLC) and AC respectively leading eventually to the activation of

PI3K and Na^+/K^+ ATPase inhibition. On the other hand, it was shown that, in alveolar epithelial cells, T3 elicited an increase in the activity and expression of Na^+/K^+ ATPase through the phosphorylation and stimulation of ERK1/2 (Lynch et al., 1986).

Parathyroid hormone (PTH) plays also a role in modulating the activity of Na^+/K^+ ATPase. In OK cells, binding of PTH to its receptor causes the stimulation of PI3K which leads to the stimulation of ERK1/2. The latter induces the activation and mobilization of PKCα towards the cell membrane where it associates with the α_1 subunit of the Na^+/K^+ ATPase facilitating its subsequent phosphorylation and inhibition (Khundmiri, Dean, Mcleish, & Lederer, 2005).

Insulin is an additional hormone known to modulate the activity of the pump. A study involving human skeletal muscle cells showed that insulin exerts a stimulatory effect on Na^+/K^+ ATPase through the MAPK signaling pathway. The activated ERK1/2 phosphorylates the α-subunit of the pump stimulating it and evoking its translocation to the plasma membrane (Al-Khalili et al., 2004). Another mechanism was also reported involving the stimulation of PI3K which in turn activates $PP\text{-}1_G$ that dephosphorylates the α-subunit of the pump leading to its activation (Ragolia, Cherpalis, Srinivasan, & Begum, 1997).

Reactive oxygen species (ROS) are also recognized regulators of the activity of Na^+/K^+ ATPase. In renal cells, hydrogen peroxide elicits oxidative stress which causes the transport of NFκB from the cytosol to the nucleus and subsequently triggers the activation of PKC. In turn, PKC induces the translocation of GPCR kinase 2 (GRK-2) to the membrane where it disconnects Dopamine 1 (D1) receptor from the G-protein coupled to it by phosphorylating the D1-G protein coupled receptor. This prevents dopamine or any D1 receptor agonist from inducing its inhibitory effect on the Na^+/K^+

ATPase (Fardoun, Asghar, & Lokhandwala, 2007). However, in renal medulla, superoxide anions, synthesized by NADPH oxidase, augment the activity of Na^+/K^+ ATPase by decreasing the amount of nitric oxide (NO) present in the cell (Bełtowski, Marciniak, Jamroz-Wiśniewska, & Borkowska, 2004).

B. Sphingosine-1-phosphate

1. Structure, Synthesis, and Degradation

Sphingosine-1-phosphate (S1P) is a biological zwitterionic molecule of lysophospholipid nature (Smith, O'Sullivan, & Gergely, 2017). It is a derivative of ceramide. Ceramide, which is composed of a sphingosine part and an acyl chain with amide linkage, can be either produced *de novo* from palmitoyl-CoA and serine in the endoplasmic reticulum or from membrane sphingolipids under the action of sphingomyelinase. Its de-acetylation by ceramidase produces sphingosine which can be changed into sphingosine-1-phosphate upon phosphorylation by sphingosine kinases (SphK1 or SphK2) (Pyne & Pyne, 2000). Like any other biological molecule, the level of S1P should be kept under control. This is achieved not only through the manipulation of the amount of S1P synthesized but also through controlling the amount of S1P molecules degraded. S1P is either reversibly dephosphorylated by S1P phosphatase back to sphingosine or irreversibly degraded by S1P lyase to produce palmitaldehyde and phosphoethanolamine (Pyne & Pyne, 2000). In addition, S1P present outside the cells can be dephosphorylated by lysophospholipid phosphatase 3 (LPP3) back to sphingosine (Escalante-Alcalde et al., 2003). Following the synthesis of S1P intracellularly, it moves to the extracellular medium with the aid of special transporters like spinster 2 and ATP-binding cassette transporters. Sphingosine kinase can also be

exported to the outside of the cell where it can synthesize S1P extracellularly. This leads to an increase in the amount of S1P in the blood which is essential for its proper functioning, for it contributes to the formation of a steep concentration gradient between blood and the interstitial fluid (Mendelson, Evans, & Hla, 2013).

In the colon, the amount of S1P produced and degraded by the respective sphingosine kinases and sphingosine lyase is related to inflammation (Suh & Saba, 2015). Under normal physiological conditions, the level of S1P in colonocytes is low and could be attributed to the low expression level of sphingosine kinases (Kawamori et al., 2008; Kawamori et al., 2005; Liang et al., 2013). Moreover, healthy intestinal cells have high S1P lyase expression and activity, contributing further to the low levels of S1P (Borowsky et al., 2012). On the other hand, during inflammation, an opposite scenario is observed. The expression level and activation of SphK1 is high in colonocytes of mice suffering from IBD and colitis-associated cancer (CAC) eventually leading to above normal levels of S1P (Maines et al., 2007; Snider et al., 2008, Chumanevich et al., 2010; Abdin, 2013). As for S1P lyase, to our knowledge, no alterations in its activity in the context of IBD and CAC have been documented; however, vitamin B6, which is a cofactor of S1P lyase, has been found at lower than normal concentrations in colon tissues during inflammation and IBD flare-up (Selhub et al., 2013; Paul, Ueland, & Selhub, 2013). Supposedly, this reduction in the cofactor would jeopardize the ability of S1P lyase to degrade S1P leading to an increase in S1P level in colonic tissue.

S1P level is augmented in colitis as well as CAC, and binds to its receptor on the membrane of intestinal epithelial cells inducing the activation of Stat3 which, in turn, promotes S1P receptor 1 (S1PR1) transcription as well as cell survival and proliferation.

Besides, S1P stimulates NFκB, which elicits the transcription of each of interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α). IL-6 stimulates Stat-3; whereas, TNF-α binds to its plasma membrane-bound receptor maintaining NFκB stimulation and thus promoting cell survival. The latter interdependent positive feedback loops are critical for the progression of intestinal inflammation into cancer and malignancy (Nagahashi et al., 2014).

2. S1P Receptors (S1PRs)

S1P present extracellulary binds to its cell-surface receptors and acts in either an autocrine or paracrine way (Xie, Liu, & Geng, 2017). S1PRs are integral heterotrimeric GPCRs made up of 7 α-helices spanning the lipid bilayer of the plasma membrane (O'Sullivan & Dev, 2013). There are 5 S1PR sub-types that differ in their expression level as well as function depending on the identity of the cell (Xie et al., 2017).

S1PR1, the product of the endothelial differential gene 1 (EDG-1), is mostly expressed in neurons where it plays a role in neurogenesis and in cells of the immune system where it influences trafficking of immune cells. It is also highly expressed in endothelial cells as well as cardiac and smooth muscle cells. Through its downstream signaling molecules, activated S1PR1 can promote cell survival as well as proliferative, migratory, and inflammatory responses (reviewed in Xie et al., 2017). For instance, it has been shown that activated S1PR1 can elicit cell migration in fibrosarcoma (Fisher et al., 2006), glioma (Yoshida et al., 2010) and Hodgkin's lymphoma (Kluk et al., 2013).

The S1PR2, also known as EDG-5 gene receptor, is predominantly expressed in neural cells of the central nervous system, cardiovascular cells, and immune cells. The prevalent expression of S1PR2 among tissues highlights its crucial role in proper cell

functioning (Xie et al., 2017). Mice displaying a reduced expression S1PR2 experience serious convulsions during the first few weeks after birth (Maclennan et al., 2001). Moreover, S1PR2 plays an indispensable role in hearing and balance (Herr et al., 2007). The specific role of S1PR2 in cancer development is still controversial and seems to be tissue specific. Mice with low expression of S1PR2 have an increase in the size of grafted lung carcinoma tumor (Du et al., 2010) while in cholangiocarcinoma, S1PR2 is involved in the tumor promoting effect of conjugated bile acids (Li et al., 2014).

The S1PR3 is vastly prevalent in the thymus, diaphragm as well as in the cardiac, brain, renal, intestinal, pancreatic, and immune cells. Functionally, S1PR3 is involved in proliferation, growth, and migration of cells (Xie et al., 2017). It has been demonstrated that deficiency in S1PR3 results in tumor growth suppression in lung adenocarcinoma as well as a halt in metastasis (Zhao et al., 2016), while S1PR3 activation supports and facilitates EGF-induced cell division (Hsu et al., 2012). In gastric cancer cells, a migratory response is initiated in response to the binding of S1P to S1PR3 (Yamashita et al., 2006).

The expression of S1PR4 (EDG-6) is limited and is restricted to blood cells including erythrocytes and lymphocytes, and its function in immunity is still not clear (Xie et al., 2017). A relatively recent study pointed out, however, to an involvement of S1PR4 in the passage of neutrophils from the circulatory system towards tissues (Allende et al., 2011). Furthermore, S1PR4 has been shown to influence the differentiation but not the function of T helper lymphocytes although it takes part in functioning of dendritic cells (Schulze et al., 2011). In addition, S1PR4 can affect the cytoskeleton in a way that allows it to regulate cell movement (Graler et al., 2003).

S1PR5 (EDG8) is mostly found in cells of the immune system as well as neurons (Xie et al., 2017). It plays an essential role in trafficking of natural killer cells towards inflammation site (Walzer et al., 2007) while in prostate tumor cells, S1PR5 is crucial for the induction of autophagy (Chang et al., 2009). Activation of S1PR5 promotes survival in oligodendrocytes (Jaillard et al., 2005).

Although all S1P receptors are coupled to G proteins, each sub-type displays a consistent scheme of G-protein types coupled to it. S1PR1 is known to bind to G_i while S1PR2 and S1PR3, couple to $G_{i,}$ G_q, and $G_{12/13}$. S1PR4 and S1PR5 couple to G_i and $G_{12/13}$ (Brinkmann, 2007).

Each type of G-protein activates different signaling pathways. G_i inhibits AC leading to a decrease in cAMP levels and PKA inhibition. The literature reports however a possibility of G_i to activate PLC pathway (Zhou & Murthy, 2004). G_i can also stimulate each of the MAPK and PI3K pathways resulting in the respective activation of ERK1/2 and Akt (Tsai et al., 2015). Signaling through G_q principally stimulates the PLC pathway while $G_{12/13}$ is associated with the activation of Rho, a small G-protein which binds to and stimulates the activity Rho-associated kinase (Brinkmann, 2007).

C. Protein Kinase A (PKA)

PKA is a serine/threonine kinase whose activity is dependent on the levels of cAMP. Structurally, an inactive PKA consists of 2 regulatory and 2 catalytic subunits bound together to form a tetrameric holoenzyme (Turnham & Scott, 2016). GPCRs modulate PKA activity. Particularly, $G\alpha_s$ AC which catalyzes the formation of cAMP from ATP. cAMP molecules bind to the regulatory subunits leading to the release of the

catalytic subunits, which when freed from the inhibitory effect of the regulatory subunits become active and phosphorylate a wide array of substrates (Taylor et al., 2008). On the other hand, $G\alpha_i$ inhibits AC and consequently, PKA is maintained in its inactive state (Sunahara, Dessauer, & Gilman, 1996). PKA specificity to a certain substrate in a distinct signaling pathway is attained through its localization in close proximity to the corresponding substrate. This is achieved with the aid of A kinase Anchoring Proteins (AKAPs) which are scaffold proteins that are connected to the dimerization/docking domain of the regulatory subunit (Taylor et al., 2008; Newlon et al., 2001; Banky et al., 2003). Moreover, PKA is regulated by means of phosphodiesterases (PDEs) which break down cAMP molecules and prevent PKA activation (Conti, Mika, & Richter, 2013).

PKA can be activated also by a cAMP independent mechanism. The catalytic subunit of PKA (PKAc) was reported to form a complex with NFκB and its inhibitor IκB, in which it is maintained in an inactive state. Disintegration of IκB by upstream molecules that are inducers of NFκB activity releases the catalytic subunit of PKA and hence restores its activity (Zhong, Suyang, Erdjument-Bromage, Tempst, & Ghosh, 1997). PKA then phosphorylates NFκB itself and increases its transcriptional activity. Another PKA regulatory mechanism that is cAMP independent is the one involving transforming-growth factor beta (TGF-β) and SMAD. TGF-β receptor activation causes the phosphorylation of Smad3 which forms a complex with Smad4. The latter activated complex interacts with the regulatory subunit of PKA releasing the catalytic subunits which become thus activated (Zhang et al., 2004). Furthermore, the RING ligase praja2, an E3 ligase, controls PKA activity through a mechanism involving ubiquitylation of the regulatory subunits and their targeting for degradation. Praja2 binds to the

regulatory subunits of PKA placing it in specific cellular locations where the kinase can perform its assigned role. When cAMP molecules bind to the PKA regulatory subunits, the catalytic PKA subunits get activated and phosphorylate the bound praja2. The phosphorylated praja2 then tags the regulatory PKA subunits with ubiquitin for degradation, thus, increasing the ratio of free catalytic to regulatory PKA subunits. As a result, PKA is maintained in an active state for a longer period and with a higher magnitude (Lignitto et al., 2011).

PKA plays a significant role in the regulation of Na^+/K^+ ATPase activity in a variety of tissues. The type of effect induced is however cell type specific (Therien & Blostein, 2000). For example, in 3T3 cells, increasing the concentration of cAMP and PKA activity had a stimulatory effect on the pump (Paris & Rozengurt, 1982) while in human kidney cells, the pump activity was lowered when phosphorylated by PKA (Braughler & Corder, 1978). A possible explanation has been proposed by Cheng et al. (1999) who showed that in rat kidney cells, intracellular calcium concentration can modulate the effect of PKA on the Na^+/K^+ ATPase. In the presence of high intracellular calcium concentration, phosphorylation of the Na^+/K^+ ATPase by PKA increased its activity while low intracellular calcium concentration evoked PKA-induced inhibition.

D. Protein Kinase C (PKC)

PKC is a serine/threonine kinase of the AGC family of kinases. Three groups of PKC isozymes were identified, depending on the cofactors needed for their activation: Conventional PKC, novel PKC, and atypical PKC. The conventional PKC isozymes include α, γ, β_I, and β_{II}, and are activated by diacylglycerol (DAG), Ca^{2+} ions, and phosphatidylserine. The novel PKC group includes δ, ε, η, and θ all of which are

stimulated by DAG and phosphatidylserine. However, the atypical PKC group, which includes the ζ and λ isozymes, is only activated by phosphatidylserine (Newton, 2001). Structurally, PKC is composed of a regulatory domain and a catalytic domain that are separated by a hinge. Members of different PKC sub-families differ in their regulatory domain. Both conventional and novel PKC regulatory domains include C1, C2, and pseudosubstrate domains. On the other hand, atypical PKC regulatory domain only comprises a C1 and a pseudosubstrate domain. The C1 domain is a binding site for phosphatidylserine, DAG, and phorbol esters in the conventional PKC, but only allows the binding of phosphatidylserine in the atypical PKC. The C2 domain is a docking site for anionic lipids but binds Ca^{2+} ion only in conventional PKC. The pseudosubstrate domain acts as an auto-inhibitor of PKC by adhering to the substrate-binding site on the catalytic domain. Conformational changes in response to certain signaling cues can cause the chopping of the hinge region connecting the regulatory and catalytic domains, leading to the detachment of the pseudosubstrate from the substrate-binding site and thus releasing the auto-inhibition on the enzyme so that it gets activated (Newton, 2001).

PKC is activated by phospholipase C (PLC) which acts on membrane phospholipids to produce DAG and inositol 1,4,5-triphosphate (IP_3). DAG induces the attachment of the inactive PKC to the plasma membrane and promotes its binding to Ca^{2+} ion and phospholipids in order to activate it. IP_3, on the other hand, impels calcium influx into the cytosol (Therien & Blostein, 2000; Nishizuka, 1995).

PKC is known to regulate the activity of Na^+/K^+ ATPase in many tissues. It has been shown to induce opposing effects on the pump depending on tissue type and experimental factors and conditions (Therien & Blostein, 2000). For instance, in kidney

cells, the concentration of intracellular Na^+ ions can play a pivotal role in determining the influence of PKC on the activity of the pump. At physiological concentrations of intracellular Na^+ ions, activated PKC elicited a rise in the activity of the Na^+/K^+ ATPase; however, this increase was gradually diminished as the intracellular concentration of Na^+ ions was augmented. Moreover, when the concentration of Na^+ ion was increased till 16 mM or higher, activated PKC induced a significant inhibition of the pump (Efendiev, Bertorello, Zandomeni, Cinelli, & Pedemonte, 2002). In another study on rodent proximal tubule, different isoforms of PKC elicited opposit effects on the Na^+/K^+ ATPase: PKC-β increased the activity of Na^+/K^+ ATPase while PKC-ζ induced pump inhibition (Efendiev, Bertorello, & Pedemonte, 1999).

E. Prostaglandin E2 (PGE2)

PGE2 is a prostanoid (Narumiya, 2009) involved in various signaling cascades. PGE2 synthesis is a multi-step process involving the enzyme cyclooxygenase (COX). The 2 COX enzymes – COX-1, which is constitutively active, and COX-2, whose activation is inducible – catalyze the conversion of arachidonic acid into prostaglandin H. Three PGE synthases can act on prostaglandin H to produce PGE2. Two of these synthases – cytosolic PGE synthase and membrane bound PGE synthase 2 - are constitutively active while the membrane bound PGE synthase 1, is inducible (Keijzer, Meddens, Torensma, & Cambi, 2013). PGE2 is degraded by the enzyme 15-hydroxyprostaglandin dehydrogenase (Tai, Ensor, Tong, Zhou, & Yan, 2002; Tai, Cho, Tong, & Ding, 2006; Tang, Yang, & Fu, 2005). PGE2 binds to its GPCRs on the surface of plasma membrane in either an autocrine or paracrine fashion. Four PGE2 receptors were identified (EP1-4) each of which can be coupled to specific G-protein sub-types.

EP-1 is known couple to G_q which stimulates PLC leading to PKC activation and an increase in cytosolic Ca^{2+} concentration (Tang et al., 2005), while EP-2 and EP-4 receptors couple to G_s and trigger the production of cAMP by AC leading to PKA activation. EP-4 can also couple to G_i inhibiting cAMP production and eventually PKA stimulation. Besides, EP-4 receptor may activate PI3K via Gi, which, in turn, provokes the stimulation of ERK1/2 (Keijzer et al., 2013; Sugimoto & Narumiya, 2007). As for EP-3, three isoforms have been identified all of which couple to G_i promoting AC inhibition (Keijzer et al., 2013).

PGE2 production can be modulated by controlling COX-2 expression by certain upstream signaling molecules and ligands. For instance, in pulmonary epithelial cells, Interleukin-1β (IL-1β) increased COX-2 expression via PKC-γ stimulation which activated the transcription factor NFκB leading to an enhanced COX-2 expression (Lin et al., 2000).

PGE2 has been shown to exert various effects in the intestine. In rabbits, it induces intestinal contractions that are crucial for the movement of food through the GI-tract by binding to EP-3 receptors of intestinal smooth muscle cells and to EP-1 receptors of myenteric neurons (Grasa, Arruebo, Plaza, & Murillo, 2006). On the other hand, binding to EP-4 receptors suppresses hypermotility in the small intestine of rats (Kunikata, Tanaka, Miyazawa, Kato, & Takeuchi, 2002), while intestinal relaxation and inhibition of intestinal contractions are induced in guinea pigs by activation of EP-2 receptors (Botella, Delvaux, Fioramonti, Frexinos, & Bueno, 1993; Shahbazian, Heinemann, Peskar, & Holzer, 2002). Moreover, PGE2 has been reported to induce mucin release through binding to its EP-4 receptor in the colon of rats as well as in the colonic cell line LS174T (Belley & Chadee, 1999). PGE2 can also protect rat intestinal

cells from lesions by coupling with its EP3 and EP-4 receptors (Kunikata et al., 2002). In rectal mucosa of patients with ulcerative colitis, PGE2 level is significantly higher than in normal individuals, suggesting an inflammatory role of PGE2 (Sharon, Liqumsky, Rachmilewitz, & Zor, 1978). Indeed, a study on T84 colonic cells has shown that extracellular PGE2 couples with its EP-4 receptors and promotes the expression of the chemokine interleukin-8 (IL-8) which in turn plays a significant role in the aggravation of inflammation (Dey, Lejeune, & Chadee, 2006; Yu & Chadee, 1998). PGE2 is suggested to have an influence on water and electrolyte excretion in cholera, for after the incubation of the rabbit intestines with either PGE2 or cholera toxin for 16 hours, water was secreted into the lumen. Furthermore, incubation of the intestines with cholera toxin resulted in an increase in the level of PGE2 in the lumen (Peterson & Ochoa, 1989).

CHAPTER III

Materials and Methods

A. Materials

FTY720P, NFκB inhibitor, Anti-EDG-1, 3, 5, 6, 8 rabbit polyclonal antibodies, and goat anti-rabbit horse radish peroxidase (HRP) conjugated IgG were purchased from Santa Cruz Biotechnology, CA, USA.

JTE-013 and CAY10444 were bought from Cayman Chemical, MI, USA

Anti p-ERK1/2 rabbit monoclonal antibody was bought from Cell Signaling, MA, USA.

Anti-ERK1/2 rabbit polyclonal antibody was purchased from Promega, WI, USA.

Phorbol-12-myrsitate-13-acetate (PMA), Calphostin C, Adenosine-3',5'-cyclic Monophosphorothioate, Rp-Isomer Triethylammonium salt (RpcAMP), and wortmannin were bought from Calbiochem, San Diego, USA.

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

Biorad assay and protein reagent, nitrocellulose membranes, and western blotting luminol reagent (Clarity western ECLsubstrate) were bought from Biorad, California, USA.

Prostaglandin (PGE2), Indomethacin, Adenosine 5'-triphosphate disodium salt (ATP), Ouabain, Dulbecco's Minimal Essential Medium (DMEM) with 4500mg/L Glucose and pyridoxine HCL, Fetal Bovine Serum (FBS), Penicillin/Streptomycin, 10x

Phosphate Buffered Saline (PBS) without magnesium and calcium, and Trypsin-EDTA were obtained from Sigma, Chemical Co, St Louis Missouri, USA.

Caco2 – human colonic adenocarcinoma cell line - was purchased from American Type Culture Collection (ATCC).

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

B. Methods

1. Caco-2 Cell Culture

Caco-2 cells were seeded in DMEM supplemented with 10% FBS, 1% penicillin (100µg/ml) and streptomycin (100µg/ml), on 100 mm culture plates at a density of 120,000 cell/ml, 1 200 000/plate , and incubated at 37°C in humidified incubator (95% O_2, 5% CO_2). At 85-90% confluence, cells were treated after an overnight starvation.

2. Protein Quantification

Proteins were quantified according to Bradford method. BSA (bovine serum albumin) standards with concentrations of 0, 0.0725, 0.145, 0.217, 0.29, 0.3625, and 0.435 µg/µl were prepared while the samples were serially diluted. In each well of the ELISA plate, 200 µl of 4 times diluted Biorad protein assay reagent were mixed with 10 µl of either a BSA standard or a diluted sample. The protein concentrations were read in an ELISA plate reader at a wavelength of 595 nm.

3. Na^+/K^+ ATPase Assay

The homogenates of the different treatments were diluted to a protein concentration of 0.5 µg/µl with histidine buffer (pH 7.4, 150 mM). Saponin (17 µl, 1% saponin) was added to the homogenates (65 µl) and the mixture was kept at room temperature for 15 minutes. Thirteen microliters of phosphatase inhibitor cocktail (300µl of pyrophosphate (200mM), 300µl glycerophosphate (200mM), and 400µl of DDW) were then added, and the mixture was incubated for an additional 15min at room temperature. The reaction was then started by incubating the homogenates with ATP and in presence of Na^+, K^+ and Mg^{2+} as indicated in the table below.

	Non-inhibited sample tube	Inhibited sample tube
NaCl (1240 mM) KCl (200 mM) $MgCl_2$ (40 mM)	10 µl 10 µl 10 µl	10 µl 10 µl 10 µl
Histidine (150 mM, pH 7.4)	20 µl	20 µl
H_2O	30 µl	0 µl
Homogenate	12 µl	12 µl
ATP (30 Mm)	10 µl	10 µl
Ouabain (15 mM)	0 µl	30 µl
Total Volume	102 µl	102 µl

Table 1: Na^+/K^+ ATPase assay: components added to each tube

Then, from each sample mixture, 12 µl were added to a buffer containing NaCl (1240mM, 10µl), KCl (200mM, 10µl), and $MgCl_2$ (40 mM, 10 µl)), histidine buffer (20 µl, 150 mM, pH 7.4), and ATP (30mM, 10 µl) in the presence or absence of ouabain (15

mM, 30 µl) and then placed in the incubator at 37°C. When ouabain was not present, it was replaced with an equal volume (30 µl) of double distilled water. After 30 minutes, 10µl 50% Trichloroacetic acid was added to each tube to stop the reaction. Samples were centrifuged (14462 xg, 5 min, room temperature). Ninety microliters of the supernatant were added to 80 µl Ferrous sulfate molybdate (0.5 mg Ferrous sulfate, 1 ml of ammonium molybdate (0.1g/L of 10N H_2SO_4), and 9 ml of DDW). The intensity of the blue color obtained is proportional to the concentration of inorganic phosphate produced. A microplate reader was used to quantify the latter concentration at a wavelength of 750 nM.

4. Caco-2 Cells Treatment:

a. Time-Response Study on the Effect of FTY720P on the Na^+/K^+ ATPase Activity

After an overnight starvation, Caco2 cells were treated with 7.5 nM FTY720P (the same concentration at which FTY720P modulated the activity of the ATPase in Caco-2 cells at 15min) for different time periods ranging from 75 to 180 minutes. For the control, an equal volume of the vehicle, DMSO, was added. Caco-2 cells were then washed with PBS buffer (pH=7.4), and scraped after addition of 60 µl of lysis buffer. The lysis buffer was prepared by the addition of 400 µl protease inhibitor (1 tablet/2ml H2O) and 100 µl of Triton-X (1mg/ml H2O) to 9.9 ml of 150 mM histidine buffer (pH=7.4). The lysed cells were homogenized for 10 seconds using PRO Homogenizer at maximum speed (around 30,000 rpm) and centrifuged for 30 min at 20000 g and 4°C. The homogenate was used to assay for the Na^+/K^+ ATPase activity and for western blot analysis, after protein quantification.

b. Identification of the S1P Receptors Involved in the Effect of FTY720P

To determine the type of S1P receptor through which FTY720P exerts its effect on the Na^+/K^+ ATPase, each of S1PR2s and S1PR3s were blocked with their respective antagonists JTE-013 (1 µM in DMSO) and CAY10444 (17.4 µM in DMF). The antagonists were added to the cells 15 min prior to FTY720P.

To further assess the involvement of S1PR2 and S1PR3, cells were treated for 2 hours with either CYM5520 (2.5 µM in DMSO) or CYM5541 (2 µM in DMSO), which are respective agonists of S1PR2 and S1PR3.

c. Involvement of PGE2 and Determination of the EP Receptors Involved

In order to assess the involvement of PGE2 in the pathway initiated by FTY720P on the Na^+/K^+ ATPase, Caco-2 cells were treated with indomethacin (100 µM, DMSO), a COX inhibitor, for 30 minutes before the addition of FTY720P. In addition, cells were treated with exogenous PGE2 (1nM in DDW, 2 hours) alone.

Agonists and antagonists were used to identify the EP receptor through which PGE2 acts. EP-3 receptor was blocked with L-798106 (10 µM, 30 minutes) and was added 30 minutes prior the 2 hr treatment with PGE2. Moreover, EP-1 and EP-3 receptors were activated by their common agonist sulprostone (1 µM for 2 hrs). As for EP-2 receptor, it was activated by its agonist – butaprost (4 µM in DMSO, 2 hrs)

d. Involvement of PKA and PKC

S1P and EP receptors can be coupled to either Gi or Gs which in turn can affect cAMP levels and PKA activity. To determine whether PKA is part of the signaling pathway, Caco-2 cells were treated with RpcAMP (30µM in DDW), a PKA inhibitor,

for 30 minutes prior to FTY720P addition. For further confirmation, cells were treated for 2 hrs with dbcAMP (10 μM in DDW), a cell permeable cAMP analogue. Similarly, since S1P and EP receptors may be bound to G_i and Gq proteins both of which can stimulate the PLC pathway activating PKC, we tested for the involvement of PKC in the signaling pathway. Calphostin C (50 nM in DMSO), which is a PKC inhibitor, was added 30 minutes prior to treatment with FTY720P. Furthermore, Caco-2 cells were treated with PMA (100nM in DMSO), which is a PKC activator, for 2 hours.

e. Involvement of Calcium

To determine whether intracellular calcium is necessary as a second messenger for the pathway initiated by FTY720P on the pump, Caco-2 cells were treated with BAPT/AM (20 nM in DMSO), a calcium chelator, for 30 minutes before treatment with FTY720P.

f. Involvement of NFκB

NFκB is known to increase the transcription of COX-2 leading to PGE2 synthesis. To examine whether NFκB is a mediator of the signaling pathway, Caco-2 cells were pre-treated with NFκB inhibitor (10 μg/ml in DDW) for 30 minutes and then FTY720P was added for 2 hours.

g. Involvement of PI3K

PI3K can be activated by S1P and EP receptors. To check whether it mediates the signaling pathway triggered by FTY720P on the Na^+/K^+ ATPase, Caco-2 cells were

treated with wortmannin (100 nM in DMSO) , an inhibitor of PI3K, for 30 minutes before the addition of FTY720P.

h. ERK1/2 Involvement

S1P and EP receptors can be coupled to Gi which in turn can promote the MAPK pathway. To determine whether ERK1/2 is playing a role in the FTY720P-induced pathway on Na^+/K^+ ATPase, Caco-2 cells were treated with PD98059 (50 μM in DMSO), which is an inhibitor of ERK1/2, 30 minutes before FTY720P addition. Each of PGE2, PKA, and PI3K can affect ERK1/2 phosphorylation and hence its activation. To examine the influence of PGE2 and PI3K on ERK1/2 activity, Caco-2 cells were treated with PGE2 (1 nM in DDW, 2 hrs) in the presence or absence of wortmannin (100 nM in DMSO, 30 minutes prior PGE2 addition). Then, western blot analysis was performed to probe for phosphorylated ERK1/2. Similarly, to investigate the impact of PKA on ERK1/2 activity, western blot analysis was done following treatment of Caco-2 cells with dbcAMP (10 μM in DDW, 2 hrs)

i. Positioning of the Involved Signaling Molecules with Respect to Each Other

To determine if PKC acts downstream or upstream of PGE2, Caco-2 cells were treated with calphostin (30 minutes) before treatment with PGE2 (2 hours). In another set of experiments, the cells were treated with PMA (100nM in DMSO, 2 hours), an activator of PKC after PGE2 synthesis was blocked with indomethacin (100 μM, DMSO) added 30 min before. Since NFκB was expected to be the transcription factor responsible for inducing COX-2 expression, we treated Caco2 cells with NFκB inhibitor (10 μg/ml in DDW) for 30 minutes prior to the 2 hour treatment with PMA or PGE2.

To examine whether PKA lies downstream PGE2, Caco-2 cells were treated with RpcAMP (PKA inhibitor, 30μM in DDW) for 30 minutes prior to PGE2 (1 nM in DDW, 2 hrs) addition. To check if PGE2 acts downstream PKA, PGE2 synthesis was inhibited by indomethacin (100 μM, DMSO) for 30 minutes before the 2 hour treatment with PKA activator - dbcAMP (10 μM in DDW). Furthermore, to position PKC and PKA with respect to each other, Caco-2 cells were incubated with RpcAMP for 30 minutes prior to treatment with PMA for 2 hours.

To determine the position of PI3K with respect to PGE2 and PKC, wortmannin (100 nM in DMSO), a PI3K inhibitor, was added for 30 minutes before the 2 hour-treatment with either PGE2 (1 nM in DDW) or PMA (100nM in DMSO), respectively.

CHAPTER IV

RESULTS

A. FTY720P Increases Na^+/K^+ ATPase Activity at 75min and Beyond

Since in HepG2 cells FTY720P modulated Na^+/K^+ ATPase activity in a time-dependent manner, we examined whether a similar trend occurs in Caco-2 cells. FTY720P exerted a time-dependent increase in the activity of Na^+/K^+ ATPase in Caco-2 cells. Maximal stimulation of the pump was observed at 90 min and was maintained high up to 3 hours (Figure 1).

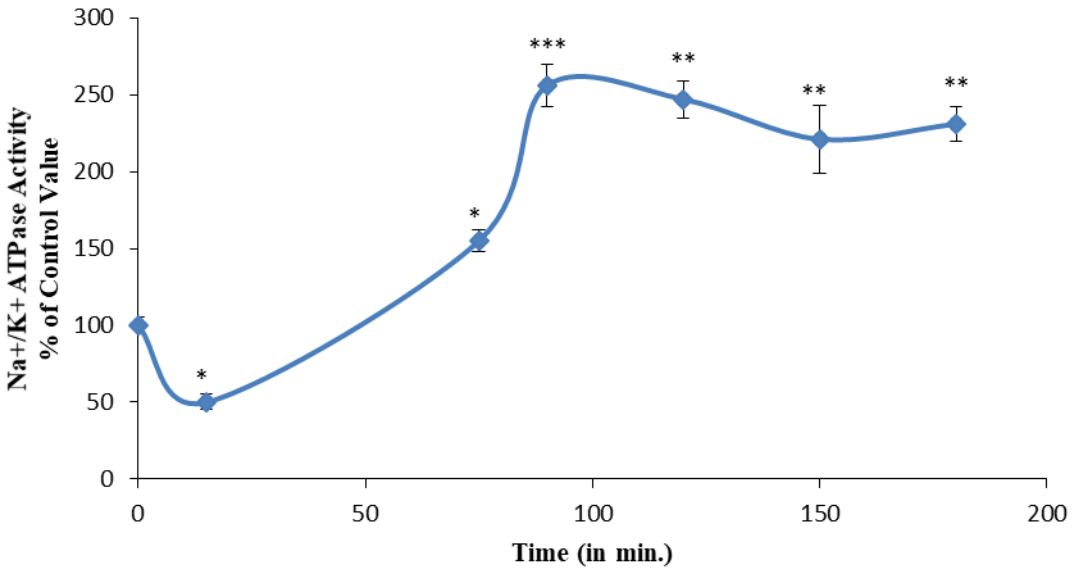

Figure 1: Time-dependent effect of FTY720P on the activity of Na^+/K^+ ATPase. Values are means ± SE. N = 3. *: different from the control $P < 0.05$; **: significantly different from the control P< 0.01; ***: significantly different from the control P< 0.001. Significant differences were tested by ANOVA followed by a Tukey-Kramer test

Consequently the two hour time point was chosen as the optimal time for FTY720P action, and was adopted in all subsequent experiments.

B. FTY720P Acts through S1PR2 to Stimulate Na^+/K^+ ATPase

Previous studies in our lab on the type of S1P receptors expressed in Caco-2 cells showed that S1PR2 and S1PR3 are the major ones expressed, with S1PR2 having the highest expression. (Rida, 2017)

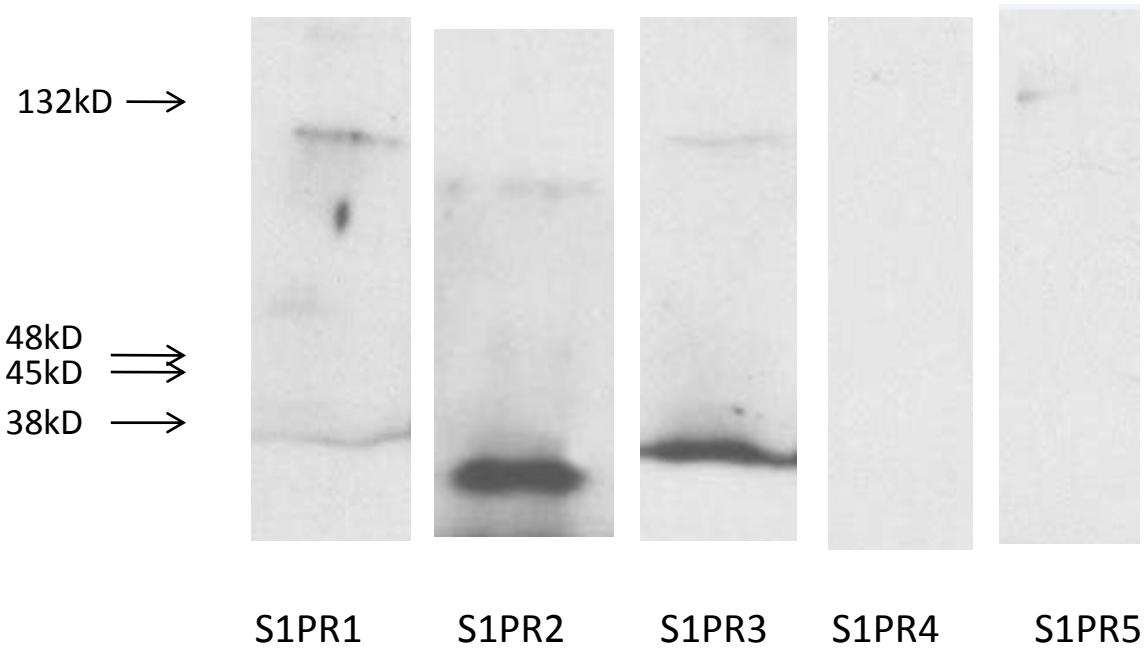

Figure 2: The expression levels of the 5 sphingosine-1-phosphate receptors in untreated Caco-2 cells. (Rida, 2017)

Since S1PR2 has the highest expression level (Rida, 2017), its involvement in the stimulatory effect of FTY720P was investigated. Blocking S1PR2 with the specific antagonist JTE-013 abolished completely the effect of FTY720P and the ATPase activity went back to control levels (Figure 3). Treating Caco-2 cells with the S1PR2 agonist CYM5520 resulted in a significant increase in the activity of Na^+/K^+ ATPase (Figure 4).

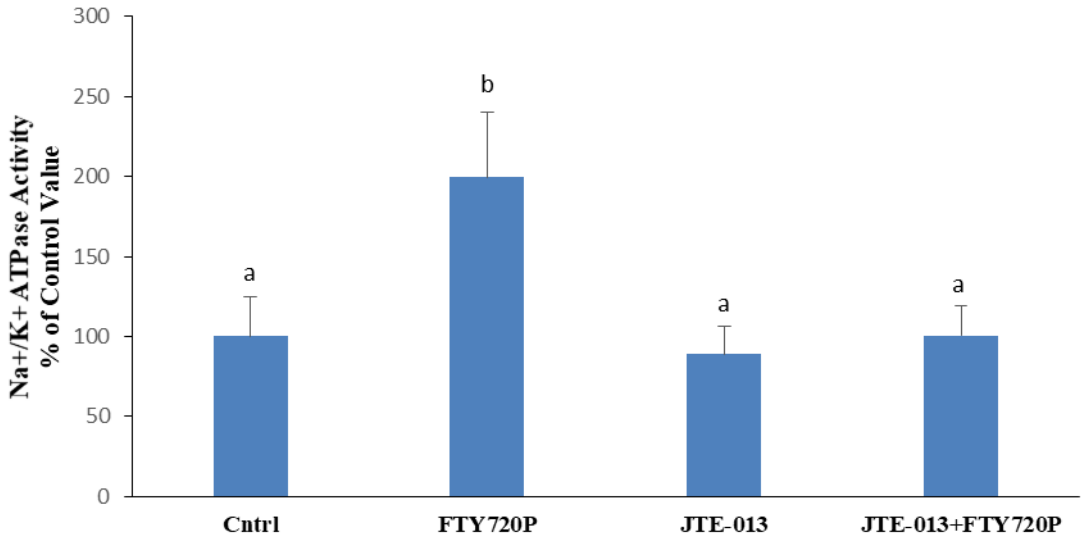

Figure 3: Effect of FTY720P on Na^+/K^+ ATPase activity in the presence of JTE-013 at 2 hours. The results are means ± SEM. N=3. Bars not sharing a common superscript are significantly different from each other at P< 0.05. Significant differences were tested by ANOVA followed by a Tukey-Kramer test.

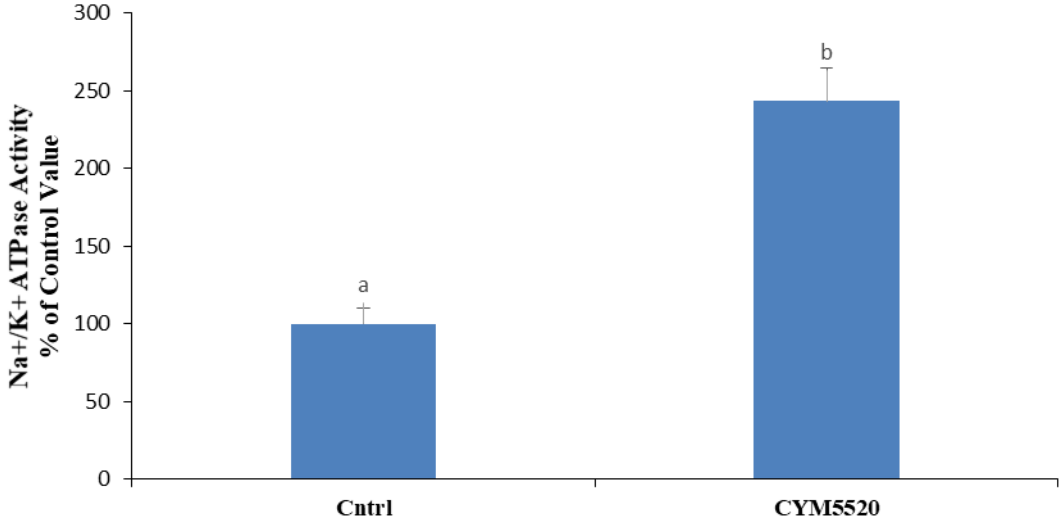

Figure 4: Effect of CYM5520 on the activity of Na^+/K^+ ATPase at 2 hours. The results are means ± SEM. N=3. Bars not sharing a common superscript are significantly different from each other at P<0.001. Significant differences were tested by t-test.

S1PR3 is also expressed in Caco-2 cells but at lower levels. In presence of the S1PR3 antagonist CAY10444, FTY720P was able to activate the pump (Figure 5). The activity of Na^+/K^+ ATPase in cells treated with CAY10444 alone (Figure 5) was not significantly different from the control. The S1PR3 agonist CYM5541 enhanced the activity of the ATPase but the increase was not significantly different from the control (Figure 6).

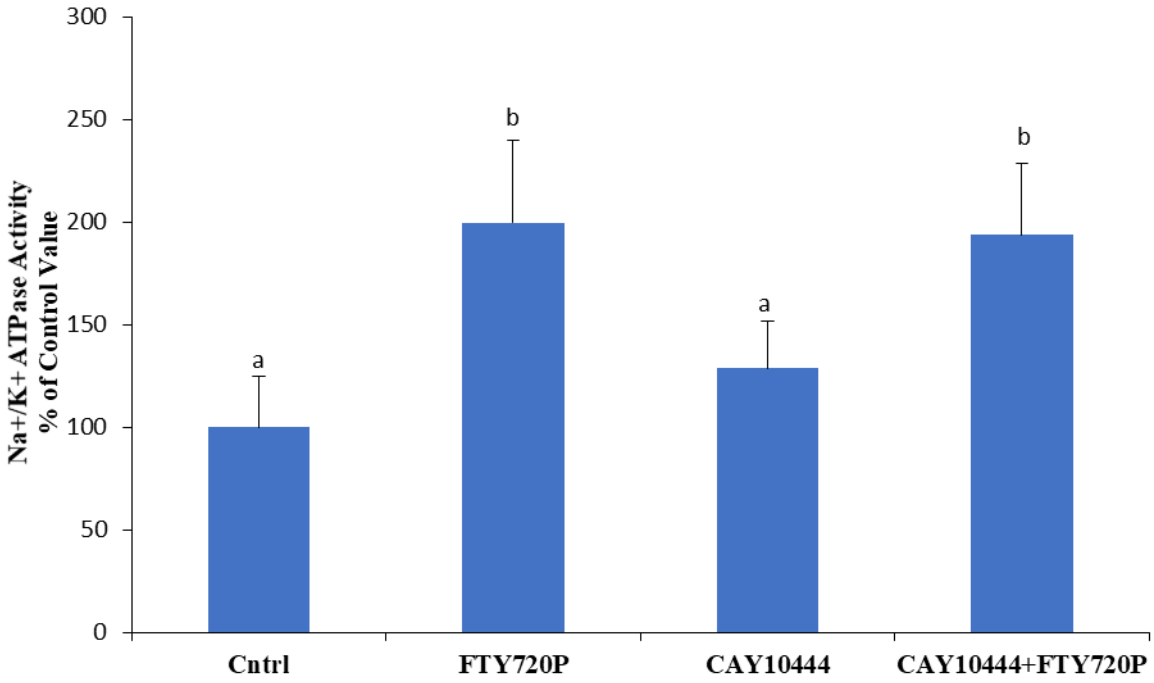

Figure 5: Effect of FTY720P on Na^+/K^+ ATPase activity in the presence of CAY10444 at 2 hours. The results are means ± SEM. N=3. Bars not sharing a common superscript are significantly different from each other at $P<0.05$. Significant differences were tested by ANOVA followed by a Tukey-Kramer test.

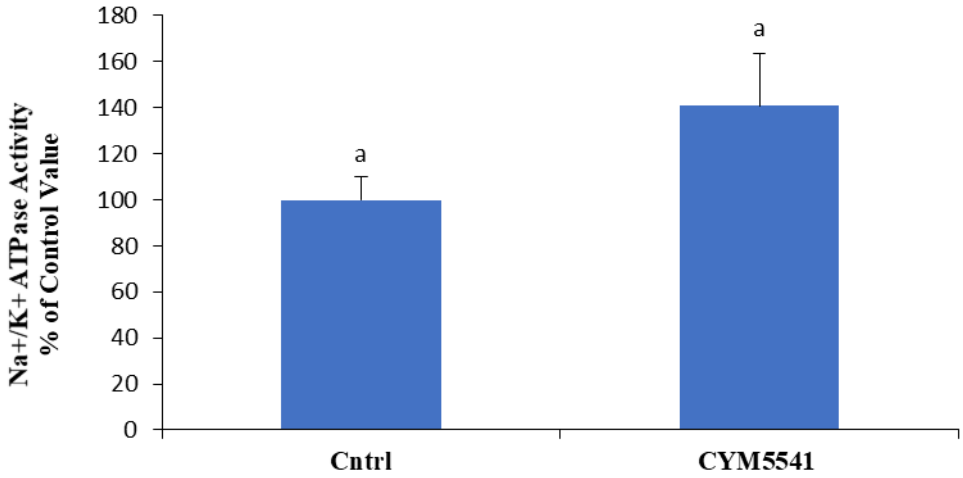

Figure 6: Effect of CYM5541 on Na^+/K^+ ATPase activity at 2 hours. The results are means ± SEM. N=3. Bars not sharing a common superscript are significantly different from each other at $P<0.05$. Significant differences were tested by t-test.

C. PKC is a Mediator in the Stimulatory Pathway Initiated by FTY720P on Na^+/K^+ ATPase

Both Gi and Gq may couple to S1PR2 triggering the PLC pathway and activating PKC. To assess whether PKC mediates the stimulatory effect of FTY720P on Na^+/K^+ ATPase, a PKC antagonist (calphostin) and a PKC agonist (PMA) were used. Calphostin abolished the effect of FTY720P (Figure 7), while PMA exerted a similar stimulatory effect on the ATPase (Figure 8)

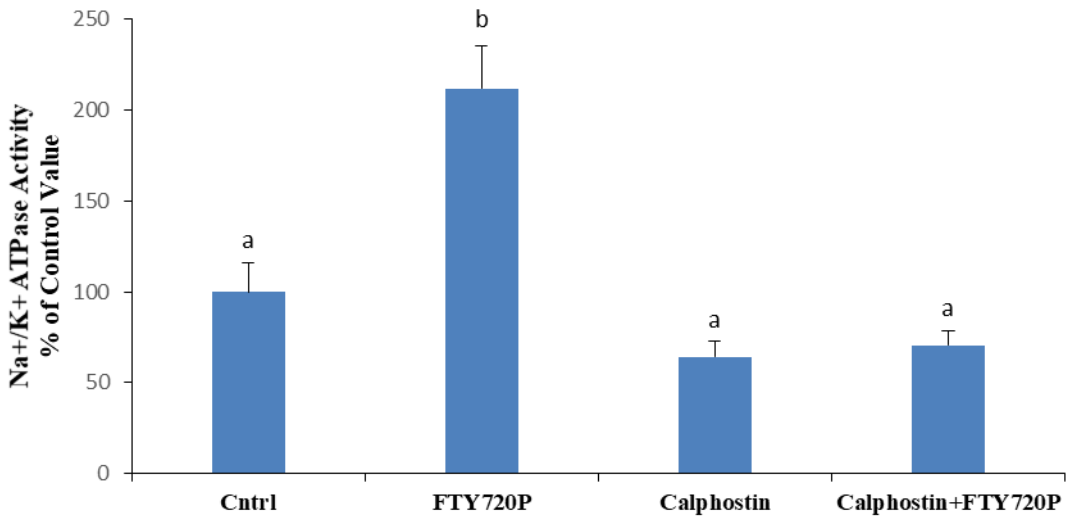

Figure 7: Effect of FTY720P on Na^+/K^+ ATPase activity in the presence of calphostin at 2 hours. The results are means ± SEM. N=3. Bars not sharing a common superscript are significantly different from each other at P<0.001. Significant differences were tested by ANOVA followed by a Tukey-Kramer test.

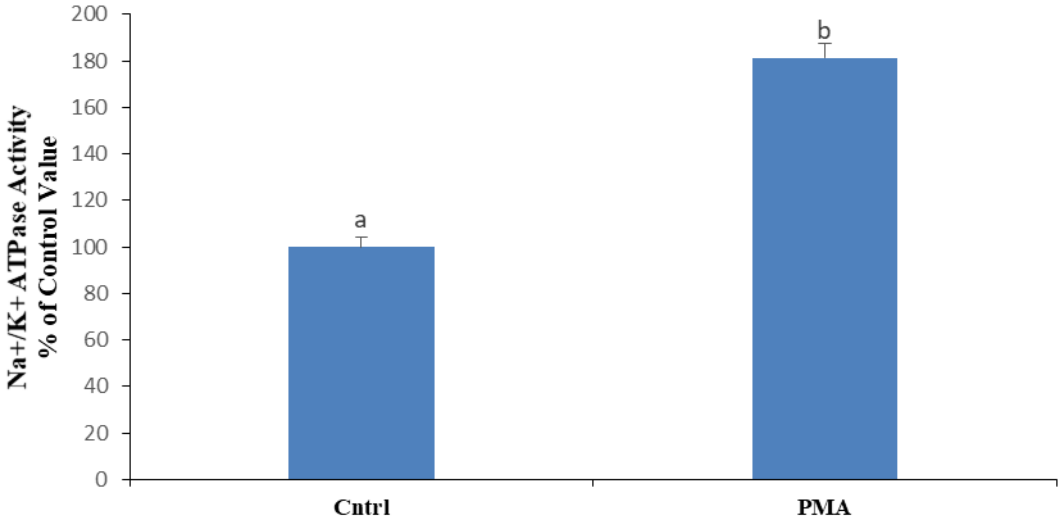

Figure 8: Effect of PMA on Na^+/K^+ ATPase activity at 2 hours. The results are means ± SEM. N=3. Bars not sharing a common superscript are significantly different from each other at P<0.001. Significant differences were tested by t-test.

D. Activation of Na^+/K^+ ATPase by FTY720P Requires Calcium

Since some types of PKCs are calcium-dependent, we examined the implication of calcium in the stimulation of the Na^+/K^+ ATPase by FTY720P, using a calcium chelator, BAPT/AM. BAPT/AM prevented FTY720P from exhibiting a stimulatory effect on the pump (Figure 9).

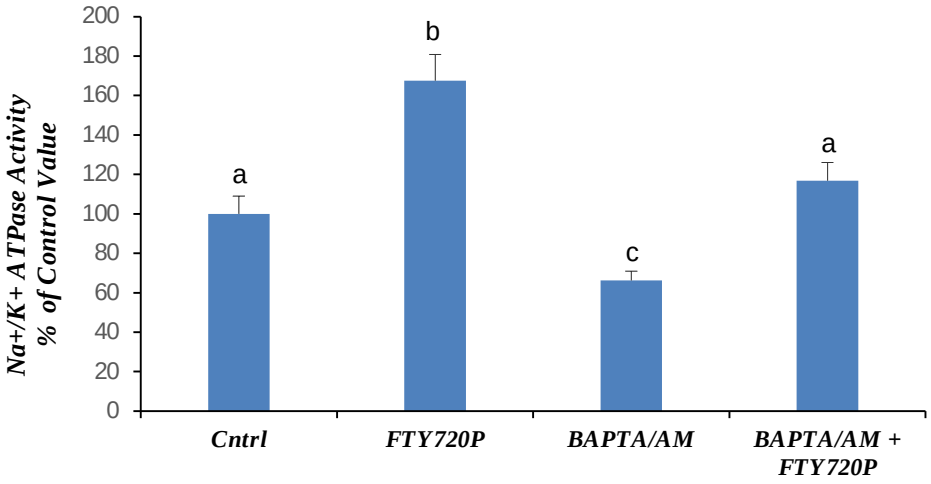

Figure 9: Effect of FTY720P on Na^+/K^+ ATPase activity in the presence of BAPT/AM at 2 hours. The results are means ± SEM. N=3. Bars not sharing a common superscript are significantly different from each other at $P<0.05$. Significant differences were tested by ANOVA followed by a Tukey-Kramer test.

E. PGE2 Synthesis is Elicited by FTY720P to Increase Na^+/K^+ ATPase Activity

PGE2 can modulate Na^+/K^+ ATPase activity. Its synthesis is catalyzed by COX enzymes. In presence of indomethacin, an inhibitor of COX enzymes, the effect of FTY720P disappeared completely (Figure 10). Furthermore, exogenous PGE2 exerted a significant increase in Na^+/K^+ ATPase activity (refer to figure 11).

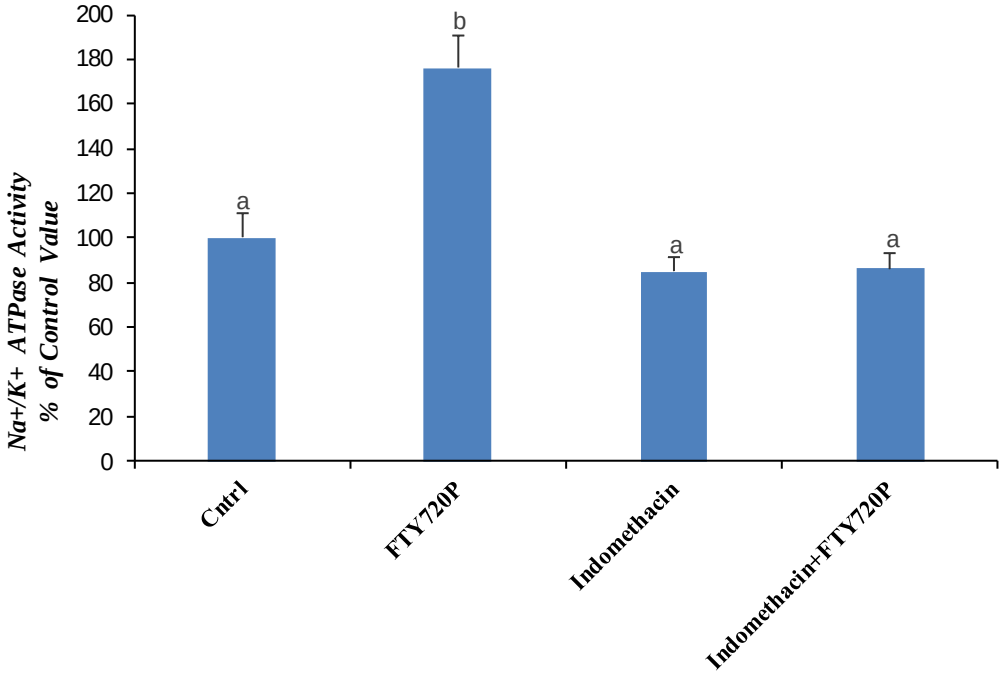

Figure 10: Effect of FTY720P on Na^+/K^+ ATPase activity in the presence of indomethacin at 2 hours. The results are means ± SEM. N=3. Bars not sharing a common superscript are significantly different from each other at P<0.001. Significant differences were tested by ANOVA followed by a Tukey-Kramer test.

F. PKC is Upstream PGE2

In the presence of calphostin, PGE2 could still induce its stimulatory effect on Na^+/K^+ ATPase (Figure 11), while PMA could not maintain pump activation in presence of indomethacin (Figure 12).

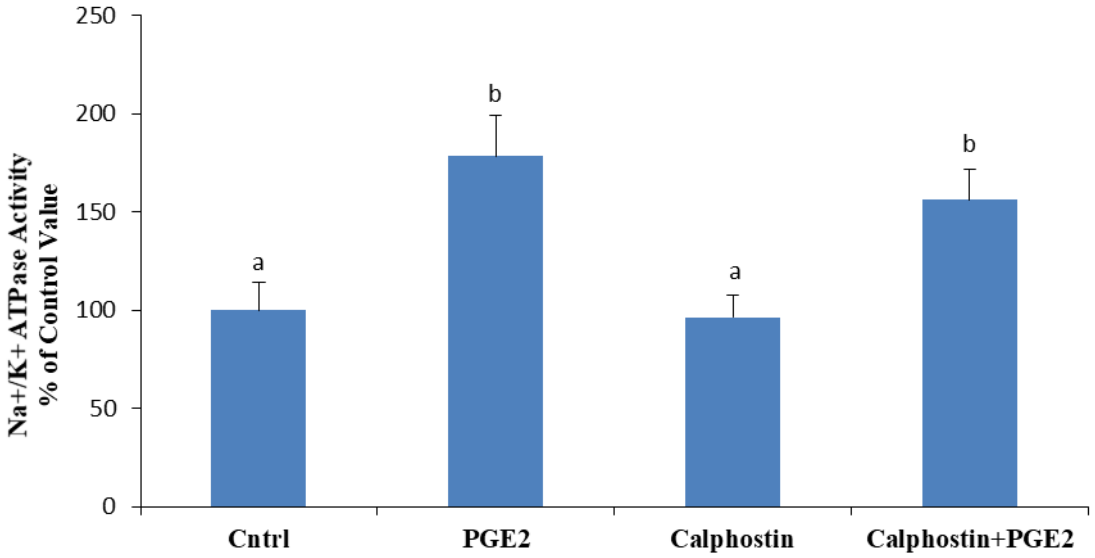

Figure 11: Effect of PGE2 on Na^+/K^+ ATPase activity in the presence of calphostin at 2 hours. The results are means ± SEM. N=3. Bars not sharing a common superscript are significantly different from each other at P<0.01. Significant differences were tested by ANOVA followed by a Tukey-Kramer test.

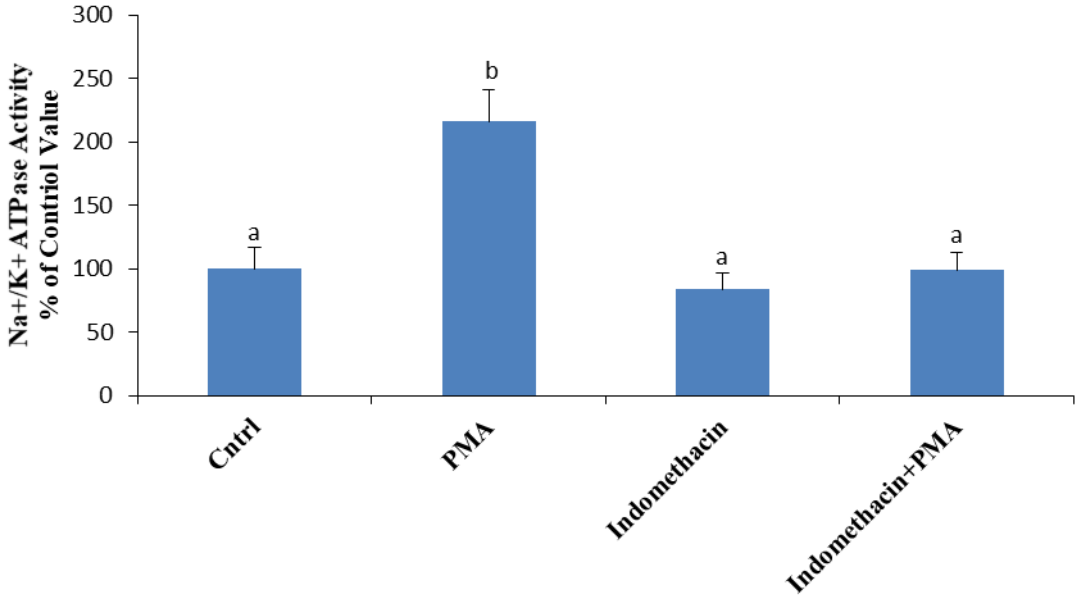

Figure 12: Effect of PMA on Na^+/K^+ ATPase activity in the presence of indomethacin at 2 hours. The results are means ± SEM. N=3. Bars not sharing a common superscript are significantly different from each other at P<0.001. Significant differences were tested by ANOVA followed by a Tukey-Kramer test.

G. FTY720P Promotes Na^+/K^+ ATPase Stimulation by Activating NFκB

NFκB is a transcription factor that is known to promote COX-2 expression leading to PGE2 synthesis. The stimulatory effect of FTY720P was not manifested when NFκB was inhibited (Figure 13).

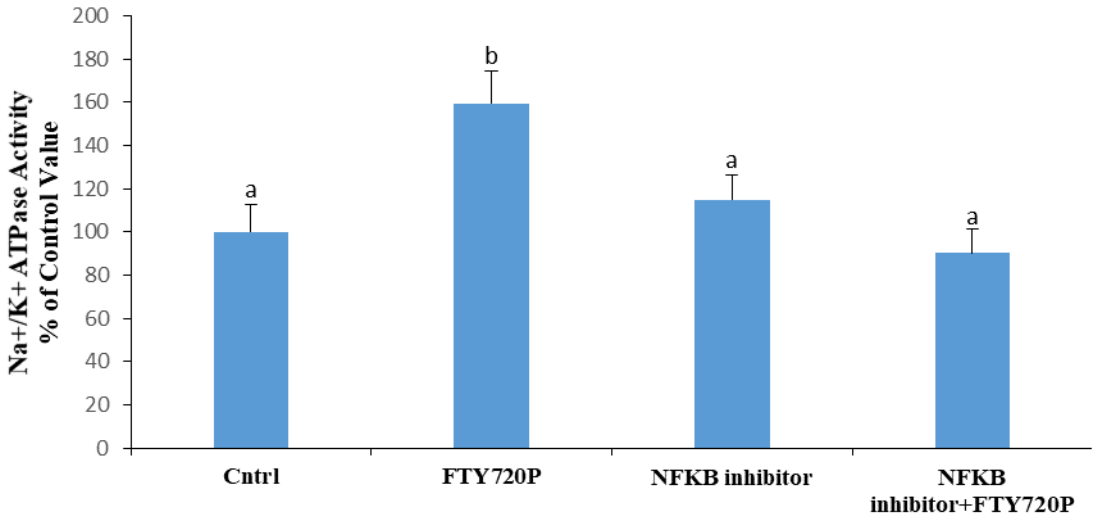

Figure 13: Effect of FTY720P on the activity of Na^+/K^+ ATPase in the presence of NFκB inhibitor. The results are means ± SEM. N=3. Bars not sharing a common superscript are significantly different from each other at $P<0.05$. Significant differences were tested by ANOVA followed by a Tukey-Kramer test.

H. NFκB Induces PGE2 Synthesis

In the presence of NFκB inhibitor, PGE2 maintained its stimulatory effect on the pump (Figure 14).

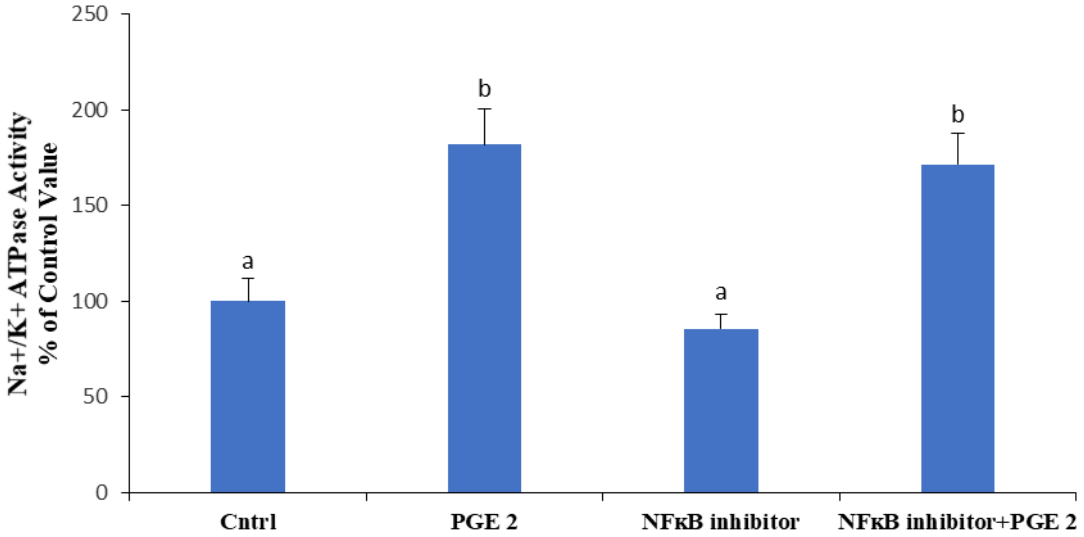

Figure 14: Effect of PGE2 on Na^+/K^+ ATPase activity in the presence of NFκB inhibitor. The results are means ± SEM. N=3. Bars not sharing a common superscript are significantly different from each other at P<0.001. Significant differences were tested by ANOVA followed by a Tukey-Kramer test.

I. PKC Activates NFκB to Increase Na^+/K^+ ATPase Activity

The stimulatory effect of PMA on the Na^+/K^+ ATPase activity was not observed in the presence of NFκB inhibitor (Figure 15)

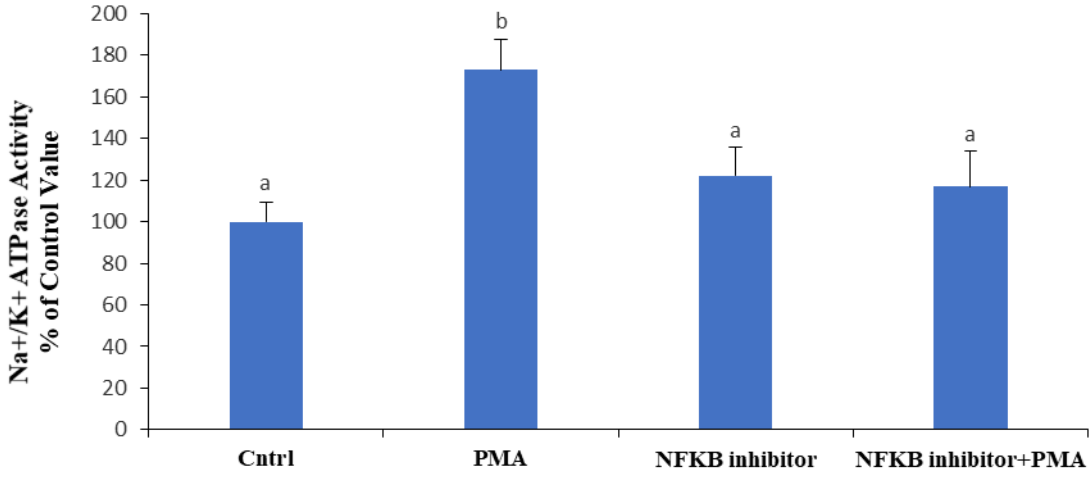

Figure 15: Effect of PMA on Na^+/K^+ ATPase activity in the presence of NFκB inhibitor at 2 hours. The results are means ± SEM. N=3. Bars not sharing a common superscript are significantly different from each other at P<0.05.
Significant differences were tested by ANOVA followed by a Tukey-Kramer test.

J. PKA Mediates the Stimulatory Effect of FTY720P on Na^+/K^+ ATPase

S1P receptors can couple to Gi and EP receptors can couple to Gi and Gs. Since the latter G-proteins modulate PKA activity, the involvement of PKA in the pathway was assessed. FTY720P was incapable of inducing its stimulatory effect on the pump in the presence of RpcAMP, a PKA inhibitor (Figure 16), while dbcAMP, a PKA activator, increased significantly the ATPase activity (Fig 17).

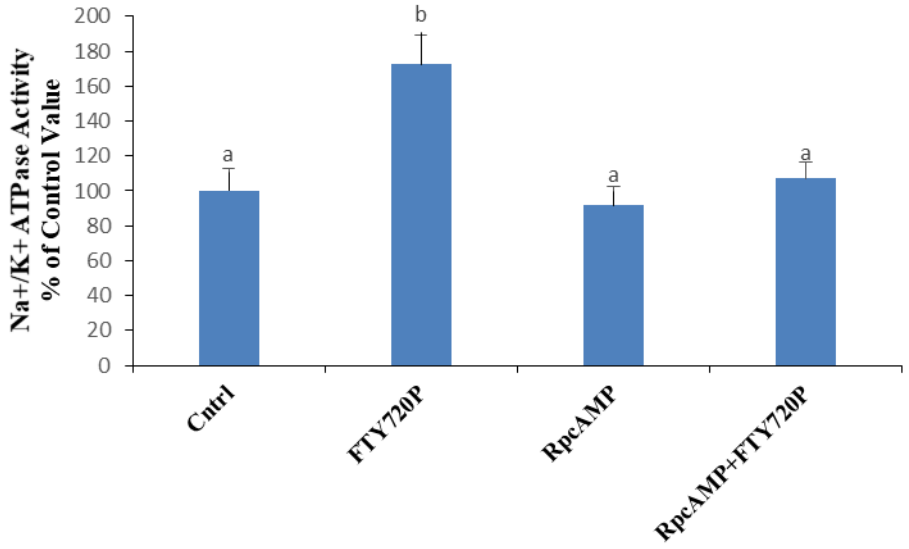

Figure 16: Effect of FTY720P on Na^+/K^+ ATPase activity in the presence of RpcAMP at 2 hours. The results are means ± SEM. N=3. Bars not sharing a common superscript are significantly different from each other at $P<0.01$. Significant differences were tested by ANOVA followed by a Tukey-Kramer test.

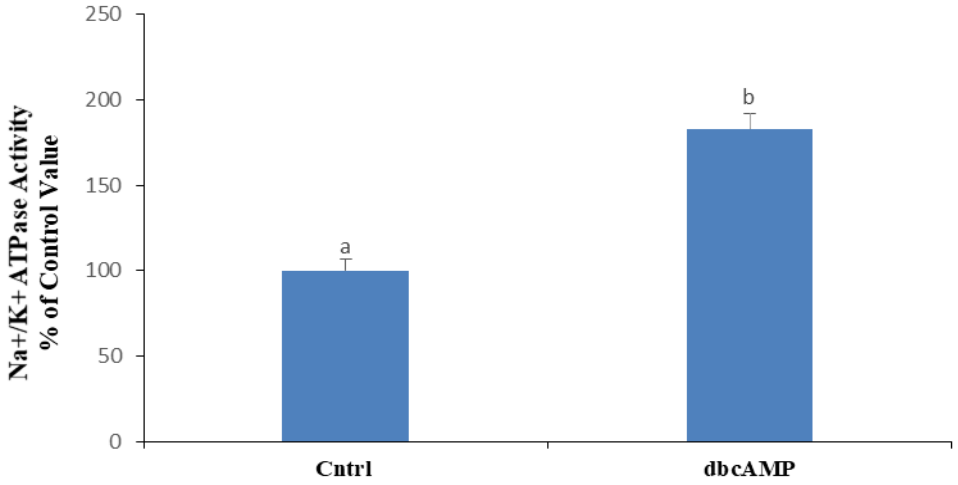

Figure 17: Effect of dbcAMP on Na^+/K^+ ATPase activity at 2 hours. The results are means ± SEM. N=3. Bars not sharing a common superscript are significantly different from each other at P<0.001. Significant differences were tested by t-test.

K. PGE2 and PKC Stimulate Na+/K+ ATPase through PKA

The PKA inhibitor RpcAMP abolished the effect of PGE2 as well as the effect of PMA, the PKC activator, on the ATPase (Figure 18).

Activating PKA with dbcAMP stimulated the Na+/K+ ATPase but not when PGE2 synthesis was blocked with indomethacin (Figure 19).

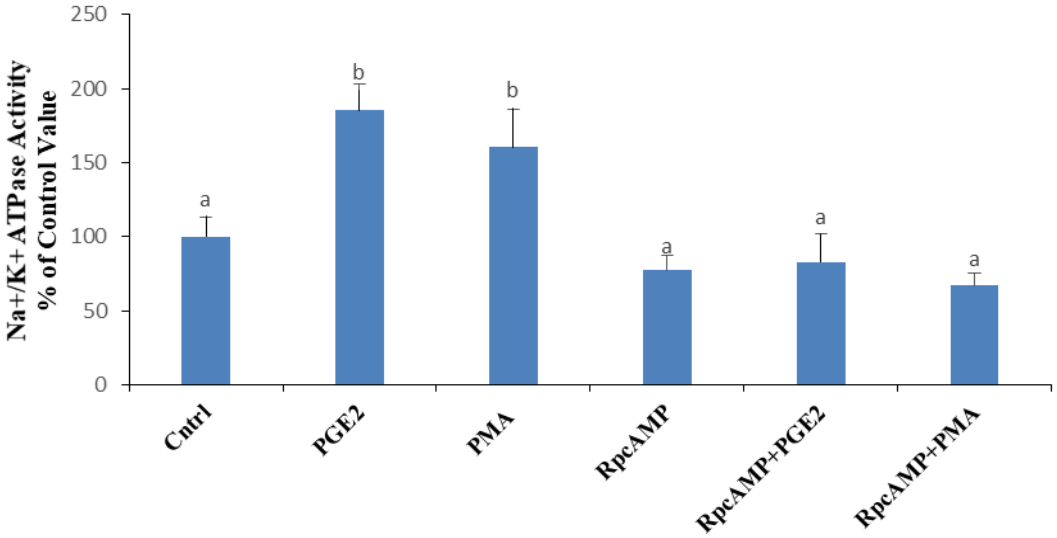

Figure 18: Effect of PGE2 and PMA on Na^+/K^+ ATPase activity in the presence of RpcAMP at 2 hours. The results are means ± SEM. N=3. Bars not sharing a common superscript are significantly different from each other at P<0.05. Significant differences were tested by ANOVA followed by a Tukey-Kramer test.

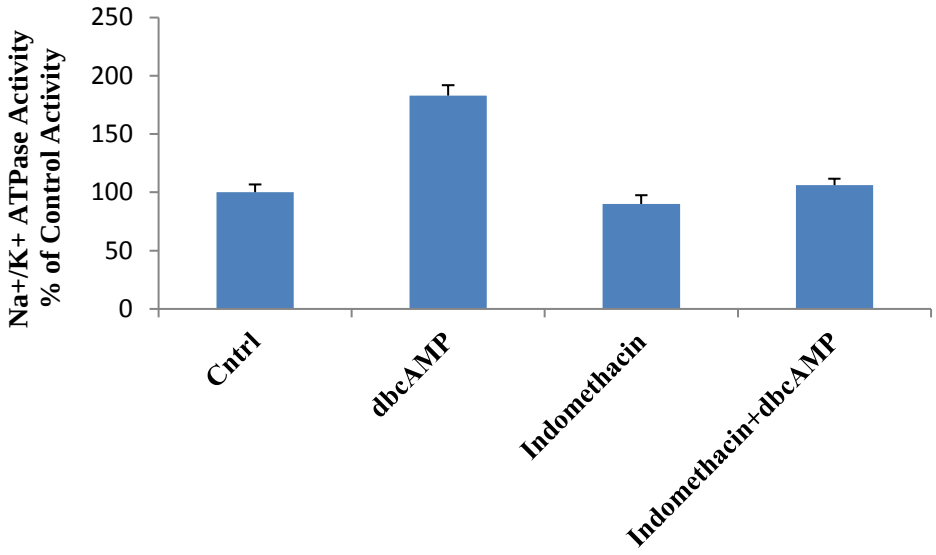

Figure 19: Effect of dbcAMP on Na^+/K^+ ATPase activity in the presence of indomethacin at 2 hours. The results are means ± SEM. N=3. Bars not sharing a common superscript are significantly different from each other at P<0.001. Significant differences were tested by ANOVA followed by a Tukey-Kramer test.

L. EP1, EP2, and EP3 Receptors do not Affect Na^+/K^+ ATPase Activity.

Blocking the EP3 receptor with L-798106, a selective inhibitor of PGE2 did not affect the stimulatory effect of PGE2 on the ATPase (Figure 20).

Sulprostone, an EP1 and EP3 agonist, as well as butaprost, an EP2 agonist, did not exert any effect on the ATPase activity (Figure 20 and 21).

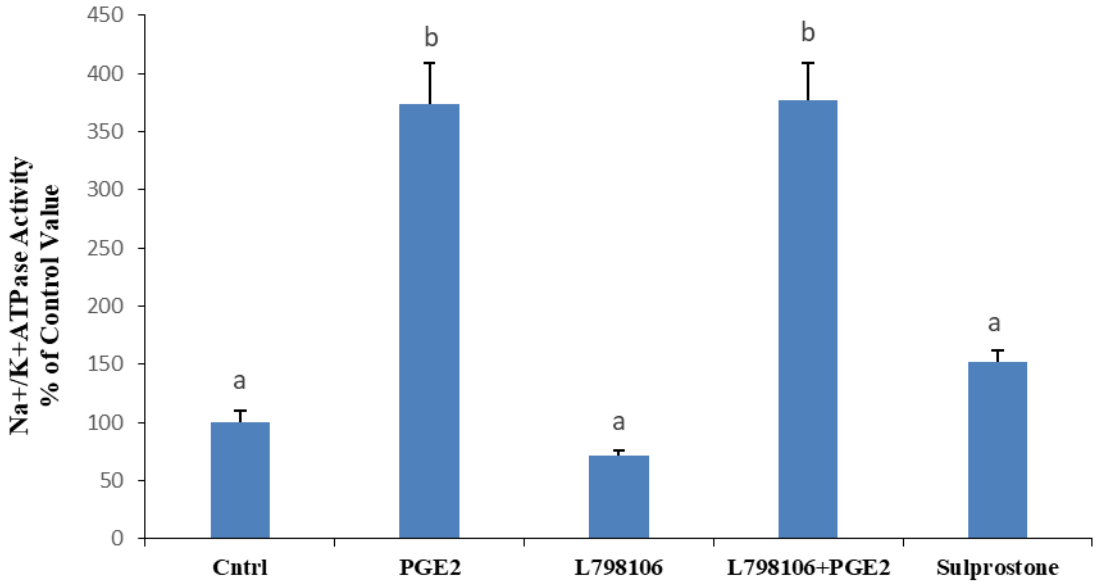

Figure 20: Effect of L-798106 and sulprostone on Na^+/K^+ ATPase activity. The results are means ± SEM. N=3. Bars not sharing a common superscript are significantly different from each other at P<0.001. Significant differences were tested by ANOVA followed by a Tukey-Kramer test.

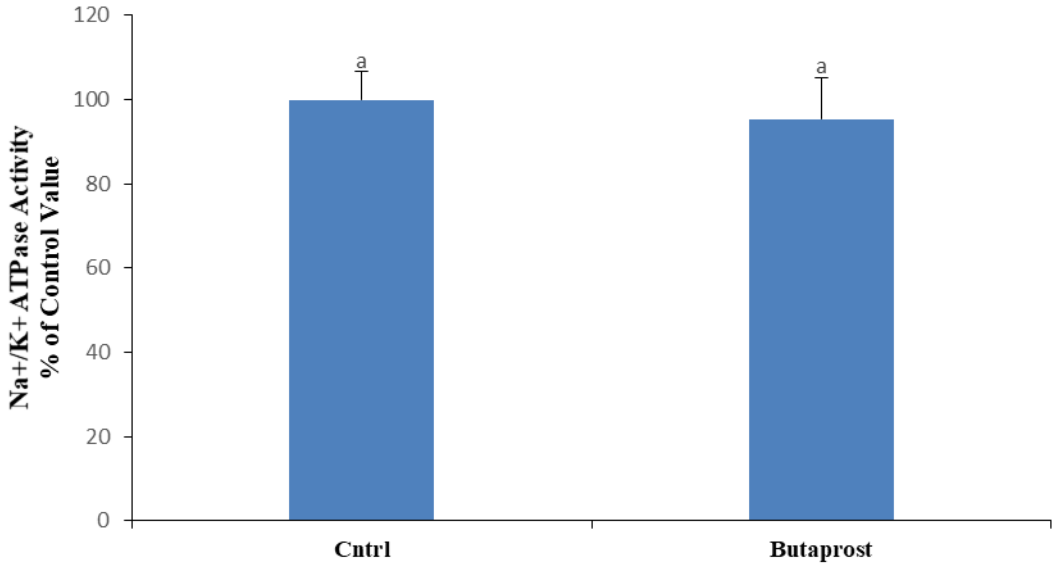

Figure 21: Effect of butaprost on Na^+/K^+ ATPase activity at 2 hours. The results are means ± SEM. N=3. Bars not sharing a common superscript are significantly different from each other at P<0.05. Significant differences were tested by t-test

M. Na^+/K^+ ATPase Stimulation by FTY720P Requires the Activation of PI3K

S1PR2, S1PR3 and EP receptors couple to Gi. PI3K/Akt pathway can be induced by Gi activation. In presence of wortmannin, a PI3K inhibitor, the stimulatory effect of each of FTY720P (Figure 22), PGE2 (Figure 23), and PMA (Figure 24) disappeared.

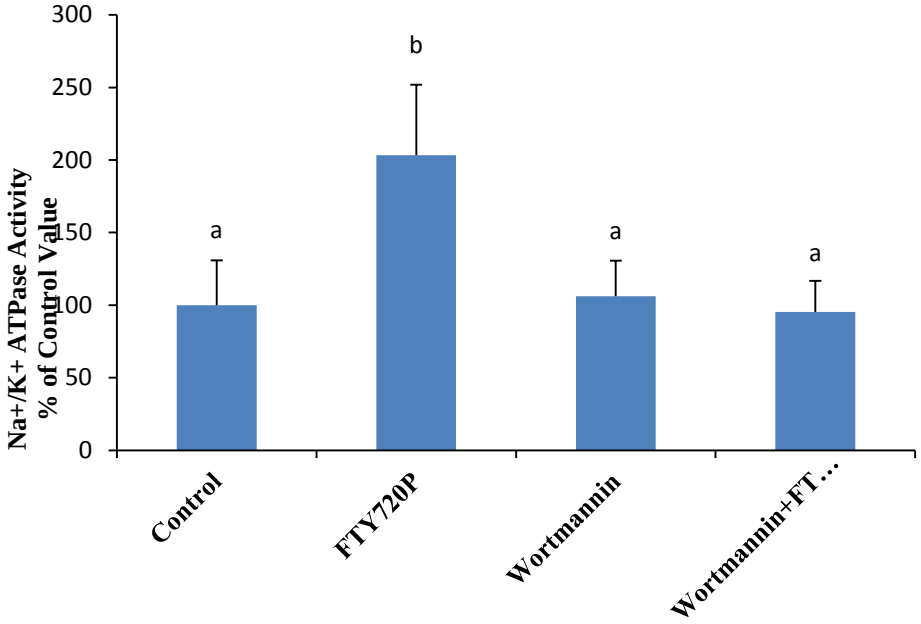

Figure 22: Effect of FTY720P on Na^+/K^+ ATPase activity in the presence of wortmannin at 2 hours. The results are means ± SEM. N=3. Bars not sharing a common superscript are significantly different from each other at $P<0.05$. Significant differences were tested by ANOVA followed by a Tukey-Kramer test.

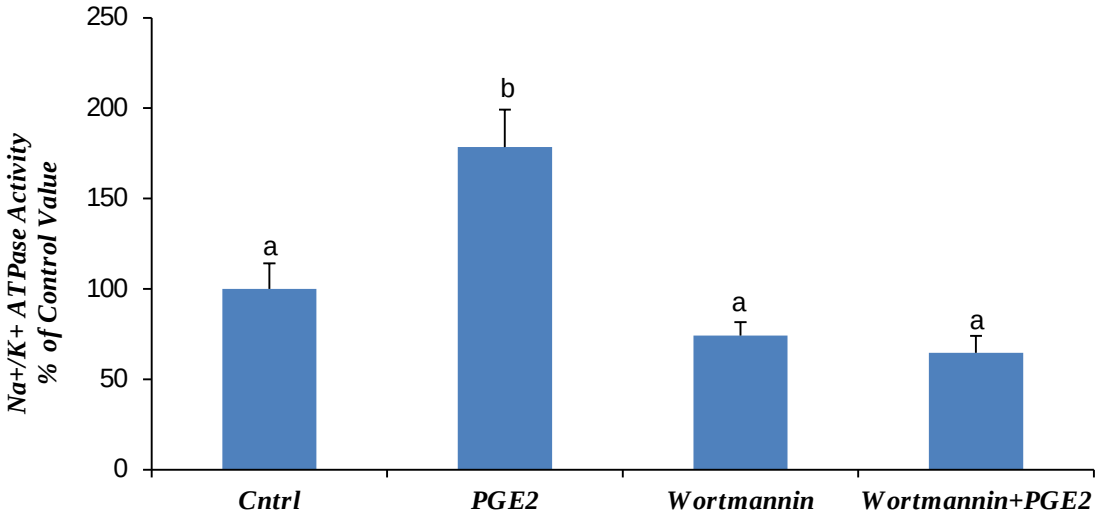

Figure 23: Effect of PGE2 on Na^+/K^+ ATPase activity in the presence of wortmannin at 2 hours. The results are means ± SEM. N=3. Bars not sharing a common superscript are significantly different from each other at P<0.01. Significant differences were tested by ANOVA followed by a Tukey-Kramer test.

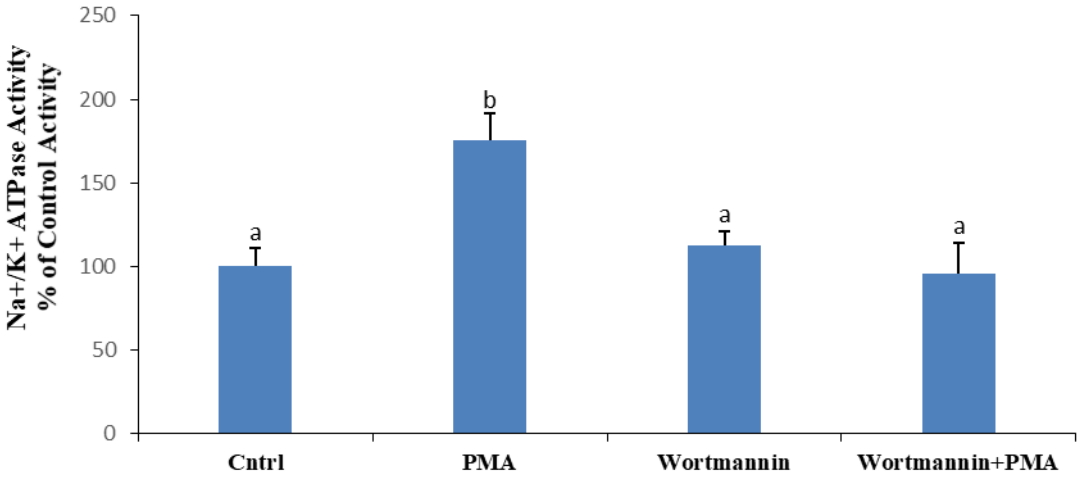

Figure 24: Effect of PMA on Na^+/K^+ ATPase activity in the presence of wortmannin at 2 hours. The results are means ± SEM. N=3. Bars not sharing a common superscript are significantly different from each other at P<0.05. Significant differences were tested by ANOVA followed by a Tukey-Kramer test.

N. PGE2 Inhibits ERK1/2 while PI3K Stimulates it

ERK 1/2 is activated by phosphorylation. Therefore, western blot analysis was performed to probe for phosphorylated ERK1/2 and check for its activation. Cells treated with PGE2 alone or the PI3K inhibitor (wortmannin) alone showed a reduced ERK1/2 phosphorylation which was reduced further in the simultaneous presence of wortmannin and PGE2 (Figures 25).

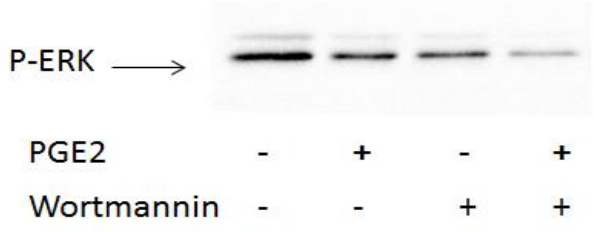

Figure 25 (a): Effect of PGE2 and wortmannin on ERK1/2 phosphorylation at 2 hours

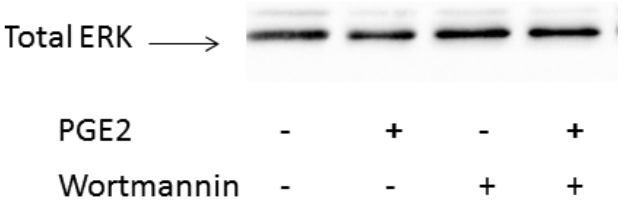

Figure 25(b): Expression level of total ERK1/2 in Caco-2 cells treated with PGE2 and wortmannin

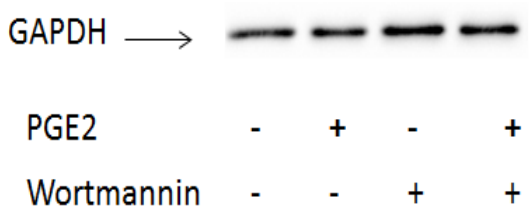

Figure 25(c): Expression level of GAPDH in Caco-2 cells treated with PGE2 and wortmannin

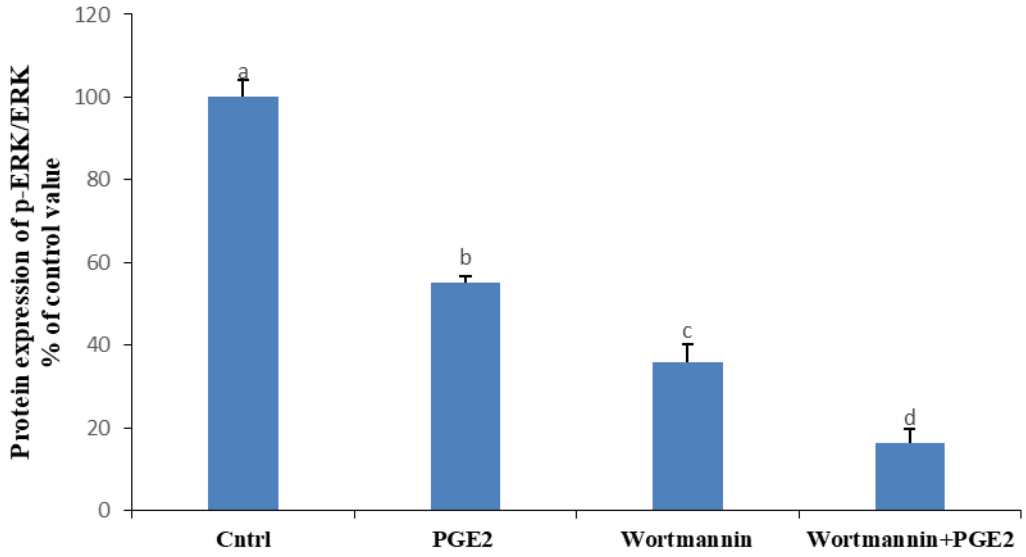

Figure 25(d): Effects of PGE2 and wortmannin on phosphorylated ERK1/2 normalized to total ERK1/2. Values are reported as arbitrary densitometry units .The results are means ± SEM. N=3. Bars not sharing a common superscript are significantly different from each other at P<0.05. Significant differences were tested by ANOVA followed by a Tukey-Kramer test.

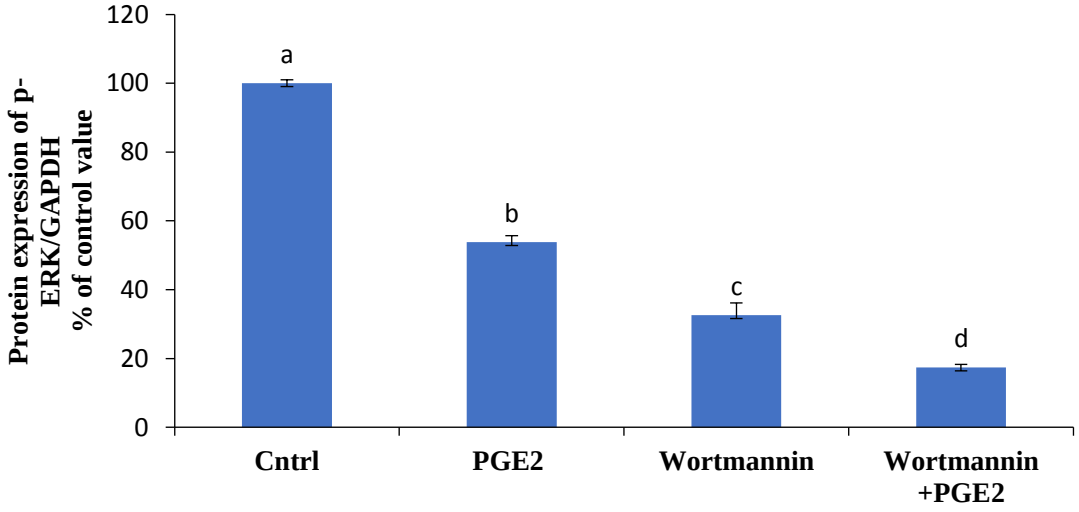

Figure 25(e): Effect of PGE2 and wortmannin on the protein expression of phosphorylated ERK1/2 normalized to GAPDH. Values are reported as arbitrary densitometry units. The results are means ± SEM. N=3. Bars not sharing a common superscript are significantly different from each other at P<0.05. Significant differences were tested by ANOVA followed by a Tukey-Kramer test.

O. PKA Inhibits ERK1/2 Phosphorylation

Since PKA is also known to influence ERK1/2 activity, western blot analysis was performed on Caco-2 cells treated with dbcAMP, a PKA activator. As indicated in the figure below, dbcAMP significantly reduced ERK1/2 phosphorylation (Figure 26).

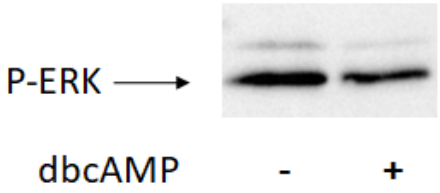

Figure 26(a): Effect of dbcAMP on ERK1/2 phosphorylation at 2 hours

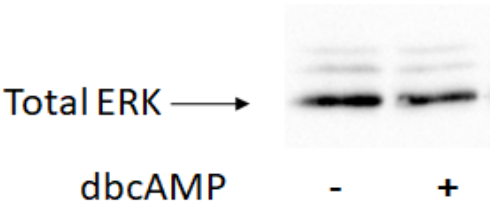

Figure 26(b): Expression level of total ERK1/2 in Caco-2 cells treated with dbcAMP

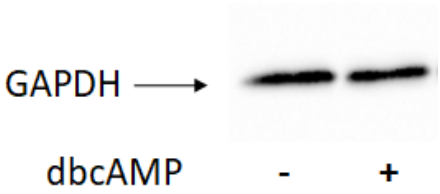

Figure 26(c): Expression level of GAPDH in Caco-2 cells treated with dbcAMP

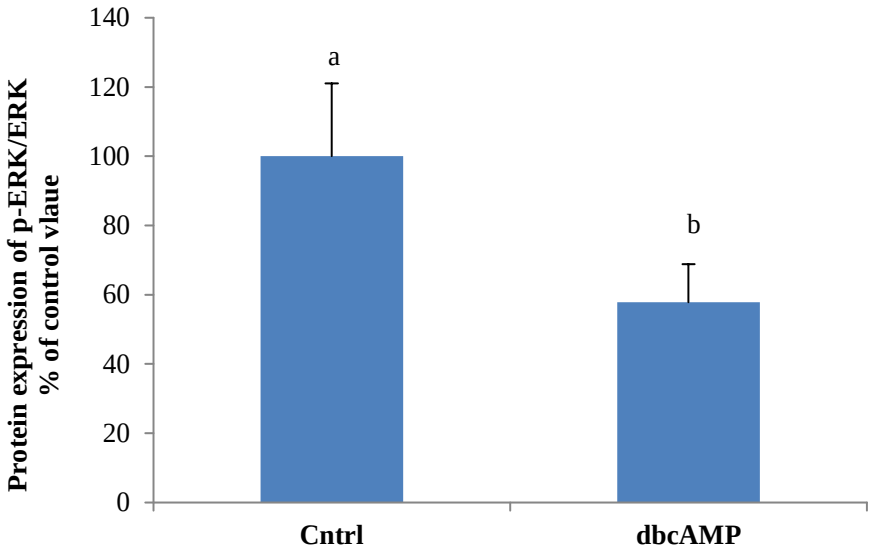

Figure 26(d): Effect of dbcAMP on the protein expression of phosphorylated ERK1/2 normalized to total ERK. Values are reported as arbitrary densitometry units. The results are means ± SEM. N=3. Bars not sharing a common superscript are significantly different from each other at P<0.05. Significant differences were tested by t test

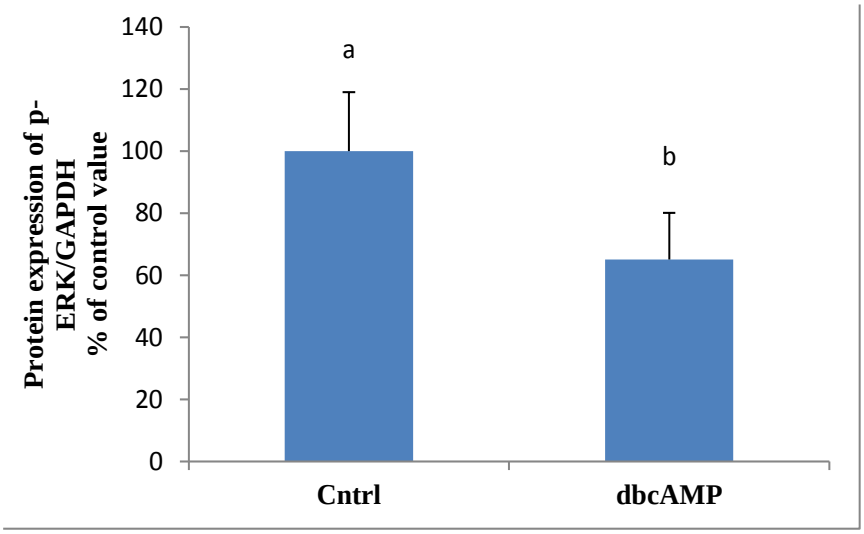

Figure 26(e): Effect of dbcAMP on the protein expression of phosphorylated ERK1/2 normalized to GAPDH. Values are reported as arbitrary densitometry units. The results are means ± SEM. N=3. Bars not sharing a common superscript are significantly different from each other at P<0.05. Significant differences were tested by t test

P. ERK1/2 is not a Mediator of the FTY720P-induced Na^+/K^+ ATPase Stimulation

Since PGE2, PI3K, and PKA, are all mediators of the signaling pathway initiated by FTY720P on the Na^+/K^+ ATPase and were shown to affect ERK1/2 activation, the involvement of ERK1/2 in the pathway was checked. In the presence of PD98059, an inhibitor of ERK 1/2, FTY720P was still capable of activating the ATPase (Figure 27).

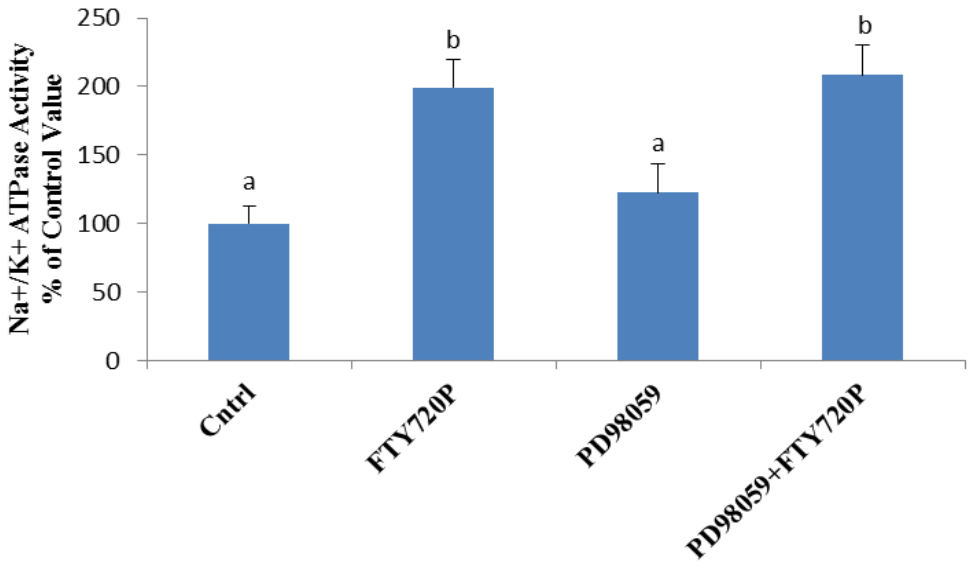

Figure 27: Effect of FTY720P on Na^+/K^+ ATPase in the presence of PD98059 at 2 hrs. The results are means ± SEM. N=3. Bars not sharing a common superscript are significantly different from each other at P<0.01. Significant differences were tested by ANOVA followed by a Tukey-Kramer test.

CHAPTER V

DISCUSSION

The Na^+/K^+ ATPase is a major transporter involved in intestinal absorption and specifically in colonic uptake of sodium and water. It is located in the basolateral membrane and creates a sodium electrochemical gradient across the apical membrane that drives the passive transport of sodium ions from the lumen into intestinal cells. Water follows by osmosis. Therefore, a change in the activity of colonic Na^+/K^+ ATPase may lead to alterations in water and electrolyte absorption in the colon that can be manifested in the form of diarrhea or constipation. Inflammatory bowel disease, whose main symptoms include diarrhea, is associated with an increase in S1P levels in colonic mucosa. Studies performed in our lab have shown that S1P mediates the down-regulatory effect of TNF-α on Na^+/K^+ ATPase in HepG2 cells. Whether S1P modulates the activity of Na^+/K^+ ATPase also in the colon is a question that we tried to address in this work, using FTY720P, an analogue of S1P. FTY720 is an oral drug prescribed for patients with multiple sclerosis. Since it has a similar structure to sphingosine, it can be phosphorylated in the body by sphingosine kinase to its active form FTY720P. Therefore, studying the effect of FTY720P on the activity of colonic Na^+/K^+ ATPase will not only uncover the effect of S1P on the pump, but will also shed light on any side effect that could be associated with the drug.

In HepG2 cells, FTY720P was shown to exert a time-dependent effect on the activity of Na^+/K^+ ATPase. A similar time-response study was conducted in Caco-2 cells to determine if a similar trend occurs in colonocytes. The results showed that

FTY720P exerts also a time-dependent modulatory effect on the activity of the Na^+/K^+ ATPase in Caco-2 cells with a maximal inhibitory effect at 15 minutes and a maximal stimulatory effect appearing at 90 min. and maintained till 3 hours. To elucidate the mechanism behind this time-dependent effect, two separate studies were conducted: one investigated the effect at 15 min while this work focused on the effect of FTY720P at 2 hours.

At 2 hours, FTY720P increased significantly the activity of the Na^+/K^+ ATPase. S1P is known to act via five different receptors: S1PR1, S1PR2, S1PR3, S1PR4, and S1PR5. Being an analogue of S1P, the same thing can be said about FTY720P. Previous work in our lab showed that S1PR2 and S1PR3 are the major S1P receptors expressed in Caco-2 cells, with S1PR2 having the highest expression. Therefore, we only tested for the involvement of the latter receptors in the signaling pathway initiated by FTY720P on the activity of Na^+/K^+ ATPase. The stimulation exerted by FTY720P was still observed in presence of CAY10444, a blocker of S1PR3 but not in presence of JTE-013 – a blocker of S1PR2 suggesting that FTY720P acts by binding to S1PR2. Further support of these findings came from the results with CYM5520 and CYM5541 respective agonists of S1PR2 and S1PR3 which showed a significant increase in the ATPase activity by CYM5520 while CYM5541 was without any significant effect on the pump. Taken together, the results suggest that FTY720P acts at 2 hours via S1PR2.

The literature reports that FTY720P can bind to all S1P receptors except S1PR2. However, this study along with several recent papers showed that FTY720P can activate S1PR2 in many cell types. For instance, in fibroblasts, FTY720P induced myofibroblast contraction (Sobel et al., 2015) via S1PR2. In another study that involves human lung fibroblasts (NHLF), FTY720P was shown to be a S1PR2 agonist (Sobel et al., 2013).

Moreover, in liver hepatocellular carcinoma (HepG2), FTY720P induced its inhibitory effect on Na^+/K^+ ATPase by activating S1PR2 (Al Alam & Kreydiyyeh, 2016).

S1PR2, as all S1PRs, is a G-protein coupled receptor. It is coupled to Gi/o, Gq, and G12/13 with every G-protein promoting a specific signaling pathway acting via different signaling molecules. Knowing that FTY720P acts via S1PR2, the next step was to determine the type of G protein involved.

A. S1PR2 and PLC Pathway

Gq is known to activate PLC leading to the production of DAG and IP3 which would activate in turn PKC (Neves, Ram, & Iyengar, 2002). On the other side, PKC has been shown to modulate Na^+/K^+ ATPase activity. Thus, PKC seems to be a potential mediator. Inhibiting PKC with calphostin abolished completely the stimulatory effect of FTY720P on the Na^+/K^+ ATPase and brought its activity back to control levels. Cells treated with PMA, a PKC activator, showed a significantly higher Na^+/K^+ ATPase activity, confirming thus the stimulatory effect of PKC on the ATPase. The results are in line with the reported effect of PKC on the Na^+/K^+ ATPase which varies depending on the type of cells and the prevailing context. In COS-7 cells, PKC phosphorylated the α_1 subunit of the Na^+/K^+ ATPase at ser-16 increasing its affinity to sodium ions and consequently its activity (Feraille et al., 2000). In intestinal cells, PKC exerted an inhibitory effect on the ATPase when activated by insulin (Serhan & Kreydiyyeh, 2011). In rat proximal tubules the PKC effect on the pump was dependent on the oxygenation levels: PKC stimulated the ATPase under high oxygen levels and inhibited it under hypoxic conditions (Feraille et al., 1995).

Three types of PKC isozymes exist, which are the conventional, novel, and atypical. The conventional type is the only one that is calcium-dependent. To determine the type of PKC involved in our pathway, the involvement of calcium ion was tested. FTY720P could not stimulate the Na^+/K^+ ATPase when a calcium ion chelator, BAPT/AM, was added, indicating that calcium is essential for the action of FTY720P and that PKC is of the conventional type. Different PKC isozymes seem to modulate differently the Na^+/K^+ ATPase. For instance, in kidney proximal tubules, PMA was reported to exert its stimulatory effect on the Na^+/K^+ ATPase through PKC-β, which belongs to the conventional PKC family and is calcium-dependent while dopamine inhibited the ATPase through PKC-ζ, which belongs to the atypical PKC family and is calcium-independent (Efendiev et al., 1999).

B. PGE2 Synthesis

The COX enzyme, which exists in two isoforms (COX-1 and COX-2), catalyzes the synthesis of PGE2 from arachidonic acid. COX-1 is constitutively expressed in tissues while COX-2 is inducible. Therefore, any change in PGE2 production will most probably be attributed to higher COX-2 expression.

Previous work in our lab demonstrated an induction of PGE2 synthesis by S1PR2 (Al Alam & Kreydiyyeh, 2016). To check if FTY720P increases similarly PGE2 production in our pathway, Caco-2 cells were incubated with FTY720P for 2 hours in the presence of indomethacin, an inhibitor of COX enzymes. FTY720P, upon suppression of PGE2 synthesis, did not have any effect on the pump's activity. Furthermore, exogenous PGE2 imitated the observed stimulatory effect of FTY720P on

the ATPase. The data suggest that FTY720P acts through S1PR2 to induce COX-2 expression and subsequent synthesis of PGE2 leading eventually to Na^+/K^+ ATPase activation.

Similar results correlating S1PR2 to PGE2 synthesis have been reported in the literature. In the Wilms' tumor cell line (WiT49), S1P was shown to induce COX-2 expression through S1PR2 leading to PGE2 production (Li et al., 2009). Moreover, in S1PR2 knockout mice, the cells of the retina displayed a subsided COX-2 expression, further suggesting a link between S1PR2 activation and PGE2 synthesis (Skoura et al., 2007). Also, in renal mesangial cells, S1P improved cell migratory ability by promoting COX-2 expression through S1PR2 (Völzke, Koch, Heringdorf, Huwiler, & Pfeilschifter, 2014).

The literature reports PKC as an upstream modulator of PGE2 synthesis (Sander & Myatt, 1990). To examine whether a similar signaling trend exists in the FTY720P induced stimulation of the Na^+/K^+ ATPase, Caco-2 cells were incubated for 2 hours with PMA (PKC activator) in presence and absence of a COX-2 inhibitor (indomethacin). PMA was unable to enhance the activity of the ATPase when PGE2 synthesis was inhibited, indicating that PKC is indeed upstream of PGE2 along the pathway. The stimulatory effect of exogenous PGE2 appeared unaltered in presence of a PKC inhibitor (calphostin), confirming the upstream position of PKC with respect to PGE2.

Many other works reported PGE2 as an effector of PKC signaling. In human peritoneal mesothelial cells, D-glucose increased PGE2 production in a PKC-dependent mechanism (Sitter et al., 1998). PKC was also reported to be needed for the interstitial

fluid flow induced increase in PGE2 synthesis (Reich & Frangos, 1993). Another study undertaken in rat thymic epithelial cells demonstrated that Phorbol-12-myristate-13-acetate (PMA), which induces tumor formation, activated Phospholipase A2 (PLA2) through PKC leading to an elevation in PGE2 production (Liu, Sun, Wen, & Hayashi, 1992).

Since COX-2 enzyme is needed for PGE2 synthesis, PKC may enhance PGE2 synthesis by enhancing COX-2 expression.

In prostate cancer, inhibition of PKC by Annexin A5 suppressed COX-2 expression (Baek et al., 2017). Similarly, in rat mesangial cells, PKC as well as PI3K were found to mediate the upregulatory effect of platelet-derived growth factor-D on COX-2 transcription. All these aforementioned studies suggest a signaling trend by which PKC is able to induce COX-2 transcription and eventually augment PGE2 synthesis (Park, Jang, & Choi, 2012).

C. NFκB

NFκB has been reported as a transcription factor needed for COX-2 transcription. To test for the involvement of NFκB, Caco-2 cells were treated with FTY720P for 2 hours in the presence of NFκB inhibitor. The inhibition of NFκB completely abolished the stimulatory effect of FTY720P on the ATPase confirming its essential role in PGE2 production.

Such a role has been reported by other works. In the spine, NFκB was found to play a significant role in promoting pain hypersensitivity after inflammation by

inducing COX-2 expression (Lee et al., 2004). Similarly IL-1β elevated PGE2 production in type II A549 cells by upregulation of COX-2 via NFκB (Newton, Kuitert, Bergmann, Adcock, & Barnes, 1997). Again, in colorectal adenocarcinoma cells, NFκB was shown to be crucial for the expression of COX-2 (Jobin, Morteau, Han, & Balfour Sartor, 1998).

In support of these results, NFκB was found to be a target of S1P action (Siehler, Wang, Fan, Windh, & Manning, 2001). In embryonic kidney cells, S1P activated NFκB by activation of the PLC pathway through Gq. In human bronchial epithelial cell line, S1P was reported to induce the secretion of IL-8 through S1PR2 and NFκB activation (O'Sullivan, Hirota, & Martin, 2014). Similarly, in endothelial cells, TNF-α promoted the S1P/S1PR2 signaling pathway leading to the stimulation of NFκB which resulted in the expression of vascular cell adhesion molecule 1 and intercellular adhesion molecule 1 (Zhang et al., 2013).

The data indicate so far that activation of S1PR2 activates PKC which in turn activates NFκB leading to PGE2 release. PKC activation of NFκB has been reported in other cells like B-cells, (Saijo et al., 2002) and neonatal rat cardiomyocytes (Rouet-Benzineb, Gontero, Dreyfus, & Lafuma, 2000). To rule out the possibility that NFκB may be enhancing the expression of another mediator downstream PGE2, Caco-2 cells were incubated with PGE2 in the presence and absence of NFκB inhibitor. Inhibiting NFκB did not alter in any way, the stimulatory effect of PGE2 on the pump suggesting that the transcription factor acts only upstream PGE2.

D. PKA

FTY720P and PGE2 bind to receptors that are coupled to G proteins and which are of the Gi or Gs type. Gi and Gs respectively inhibit or stimulate adenylyl cyclase leading to a decrease or increase in cAMP levels and consequently a decrease or increase in PKA activity. On the other hand, PKA is known to regulate Na^+/K^+ ATPase activity (Cheng et al., 1997). Therefore, PKA was suspected to be a mediator in the signaling pathway. When PKA was inhibited with RpcAMP, FTY720P could not exert its stimulatory effect, and the activity of the ATPase was restored to control levels, indicating that the observed stimulation is due to PKA activation. In support of this conclusion is the increase in activity observed upon treating the cells with dbcAMP, a PKA activator. The results are in line with what was reported in literature concerning the tissue dependent modulatory effect of PKA on the Na^+/K^+ ATPase. For instance, in rat brain, cAMP was shown to reduce Na^+/K^+ ATPase activity (Lingham & Sen, 1982) while in liver cells (Bradford, Hayes, & Mcgivan, 1985) and proximal convoluted tubules (Breton, Beck, & Laprade, 1994), cAMP exerted a stimulatory effect. .

Since both FTY720P and PGE2 can activate PKA through Gs, PKA can be upstream or downstream of PGE2. Upon inhibition of PKA, the stimulatory effect of PGE2 on the Na^+/K^+ ATPase was completely suppressed indicating that PKA acts downstream PGE2. If PKA is upstream PGE2, then blocking PGE2 synthesis with indomethacin would be expected to prevent PKA from exerting its stimulatory effect on the ATPase. This is not what was observed when Caco-2 cells were treated with dbcAMP, an activator of PKA, in presence of indomethacin. The results indicate that PGE2 acts through an activation of PKA.

After thoroughly examining and confirming the involvement of PKC and PKA in the signaling pathway initiated by FTY720P on Na^+/K^+ ATPase, the type of G protein through which S1PR2 exerts its stimulatory effect on the ATPase can be identified. S1PR2 is known to be coupled to Gi, Gq, and G12/13. Gq proterins are known to activate the PLC pathway whereby phospholipase C catalyzes the production of diacylglycerol (DAG) and inositol 1,4,5-triphosphate (IP3) from phosphatidylinositol 4,5-bisphosphate (PIP2), which is a phospholipid found in plasma membranes. In turn, DAG activates PKC while IP3 increases intracellular calcium concentration. On the other hand, Gi proteins inhibit adenylyl cyclase through the α-subunit (Gαi) leading to a decrease in cAMP levels and subsequent decrease in PKA activity (Neves et al., 2002). PKC and PKA were shown to be mediators in the signaling pathway. PKA is activated and not inhibited; therefore, the involvement of Gi can be ruled out. Furthermore, upon PKC inhibition, the stimulatory effect of FTY720P on the ATPase was completely abrogated thus indicating that only Gq proteins are involved. Since inhibition of PKC brought the activity levels of the ATPase to control values, this eliminated the possibility that FTY720P could be acting through another different G-protein sub-type.

E. PGE2 and EP Receptors

Four EP (EP1-4) receptor sub-types have been identified, all of which are G-protein coupled receptors. To identify the EP receptor sub-type responsible for inducing the stimulatory effect on the Na^+/K^+ ATPase, each EP receptor was tested at a time.

EP1 receptor is known to be coupled to Gq (Tang et al., 2005) which in turn induces the PLC pathway activating PKC. However, when PKC was inhibited by

calphostin, the stimulatory effect of PGE2 on the Na^+/K^+ ATPase was not affected in any way suggesting that PKC does not act downstream PGE2. Hence, the EP receptor involved does not act through PKC and is not coupled to Gq. Therefore, most probably, PGE2 is not inducing its stimulatory effect on the ATPase through EP1. To confirm the latter conclusion, caco-2 cells were treated with sulprostone, which is an EP-1 and EP-3 agonist. Activating EP-1 receptor did not alter Na^+/K^+ ATPase activity, thus supporting the previous result and confirming that PGE2 does not act through EP1 receptor to activate the ATPase.

On the other hand, EP2 receptor is coupled to Gs which is known to activate PKA by increasing cAMP synthesis (Keijzer et al., 2013). Upon inhibition of PKA with RpcAMP, the stimulatory effect of PGE2 on the activity of Na^+/K^+ ATPase was completely abrogated thus indicating that PKA lays downstream PGE2 along the pathway. This suggested that PGE2 might act through EP2 to stimulate the ATPase. However, upon treating caco-2 cells with butaprost, which is an EP2 agonist, the activity of Na^+/K^+ ATPase was not augmented inferring that PGE2-induced ATPase activation does not involve EP2 receptor.

As for EP3, it is known to be coupled to Gi proteins which inhibit PKA. If PGE2 acts via PKA inhibition, then a similar stimulatory effect of the ATPase should be observed when PKA is inhibited with RpcAMP. Since this was not the case, the involvement of EP3 receptors was ruled out. Indeed, upon treating caco-2 cells with sulprostone, which is an EP3 agonist, activated EP3 did not alter Na^+/K^+ ATPase activity suggesting that PGE2 does not increase the activity of the ATPase through EP3. Upon inhibition of EP3 by L798106, PGE2 was still capable of inducing Na^+/K^+

ATPase stimulation, further confirming that EP3 is not involved in the stimulatory pathway elicited on the ATPase.

Since all other EP receptors were shown to be uninvolved in the signaling pathway, EP4 receptor is the one through which PGE2 acts in order to activate the Na^+/K^+ ATPase.

EP4 can be coupled to Gs and Gi (Keijzer et al., 2013). Since PGE2 was shown previously to activate PKA, then it acts through EP4 receptors that are coupled to Gs.

F. PI3K

S1P has been reported to modulate PI3K activity (Liu, Liu, Liu, Zhang, & Lin, 2016), and PI3K, in turn, is known to regulate the activity of Na^+/K^+ ATPase. Therefore, PI3K was suspected to mediate the action of FTY720P on the ATPase. Inhibiting PI3K with wortmannin totally prohibited FTY720P from exerting its stimulatory effect, indicating that PI3K kinase is an essential mediator and that no other pathway exists in addition to this pathway. Had another pathway been involved then still a partial stimulation would be observed, when PI3K is inhibited. Modulation of Na^+/K^+ ATPase by PI3K has been addressed in several papers and the effect of the latter kinase seems to be tissue-dependent. In intestinal epithelial cells, PI3K mediated the inhibitory effect of insulin on the activity of Na^+/K^+ ATPase (Serhan & Kreydiyyeh, 2011). Furthermore, in the proximal tubule cells of the kidney, PI3K was shown to be crucial for the gastrin-induced phosphorylation and internalization of Na^+/K^+ ATPase (Liu, Konkalmatt, Yang, & Jose, 2016). On the other hand, PI3K along with Src kinase were shown to be required for the 3,3′,5-Triiodo-L-thyronine-induced stimulation and

expression of Na^+/K^+ ATPase in epithelial cells of the alveoli (Lei, Mariash, & Ingbar, 2004).

Since each of PKC, PGE2, and PI3K were shown to be implicated in the pathway and since several studies have demonstrated and discussed the cross-talk among PI3K/Akt, PLC and PGE2 signaling pathways, the position of PI3K with respect to PKC and PGE2 had to be elucidated. The inhibition of PI3K with wortmannin completely and independently prevented PMA – a PKC activator – as well as PGE2 from exerting their stimulatory effects on the Na^+/K^+ ATPase. This indicates that PKC and PGE2 are inducing the activation of the ATPase through PI3K. However, it has already been shown that PKC is upstream PGE2; thus PKC is activating PI3K through PGE2. Such an effect of the prostaglandin on PI3K is supported by many studies conducted in various tissues. For instance, PGE2 was reported to stimulate the PI3K/Akt pathway leading to cancer cell proliferation in colonocytes (Castellone, Teramoto, Williams, Druey, & Gutkind, 2005). In addition, PGE2 produced in response to TGF-β-induced COX-2 expression promoted prostate cancer cell invasion and migration through PI3K/Akt pathway (Vo et al., 2013).

So far it has been demonstrated that FTY720P binds and activates S1PR2 and stimulates Gq protein to which it is coupled. Gq, in turn, induces the PLC pathway activating PKC. PKC, then, activates the transcription factor NFκB which induces the expression of COX-2. COX-2 catalyzed the synthesis of PGE2 which binds to and activates EP4 receptor, leading to Gs activation and stimulation of PKA and PI3K. Since EP4 receptors couple to Gs and since only one pathway exists below EP4, PKA should be the most upstream mediator underneath Gs. A similar study was conducted in caco-2 whereby PGE2 affects apoptotic pathway through EP4 receptor, PKA, and PI3K

with both PKA and PI3K being downstream EP4, and PKA being upstream PI3K (Leone et al., 2007). Besides, in the ovary, PKA stimulated by Follicular stimulating hormone (FSH) through a GPCR was shown to induce the activation of PI3K (Law, White, & Hunzicker-Dunn, 2016). Furthermore, PI3K/Akt pathway gets activated by PKA upon stimulation of glucagon-like receptor 1 in bone marrow stromal cells (Meng et al., 2016).

G. ERK1/2

ERK1/2 has been reported in the literature as a downstream effector of PGE2 and PI3K (Yen, Kocieda, Jing, & Ganea, 2011). Since both PGE2 and PI3K were shown to mediate the FTY720P-induced stimulatory effect on Na^+/K^+ ATPase, ERK1/2 was also suspected as a mediator. For this purpose, the effect of PI3K and PGE2 on ERK1/2 was studied using western blot analysis to probe for phosphorylated ERK1/2 (p-ERK1/2), which is the activated form of ERK1/2. Inhibiting PI3K with wortmannin reduced the expression of p-ERK1/2 suggesting that PI3K activates ERK1/2. On the other hand, treating caco-2 cells with PGE2 led to a decrease in the expression level of p-ERK1/2 indicating that PGE2 inhibits ERK1/2. Inhibiting PI3K in the presence of PGE2 showed a higher reduction in p-ERK1/2 expression than the one observed with PGE2 alone or wortmannin alone. The stimulatory effect of PI3K on ERK is expected to be lost upon its inhibition. The results suggest the presence two distinct pathways affecting ERK activity. One pathway activates ERK via PI3K and the other inhibits ERK through mediators that still need to be determined. The inhibitory pathway is

dominant over the stimulatory pathway, and this is why the net effect of PGE2 on ERK is inhibitory.

Since PKA has also been reported as an upstream effector of ERK1/2 (Yen et al., 2011) and since it was shown to be downstream PGE2 in the FTY720P-induced signaling pathway, its effect on ERK1/2 activation was studied using western blot analysis. Treating caco-2 cell with the PKA activator – dbcAMP – resulted in a significant reduction in ERK1/2 phosphorylation thus affirming the inhibitory effect of PKA on ERK1/2. Since PKA is shown to be activated by PGE2 and because PGE2 is acting on ERK1/2 through two pathways, it seems that PKA lies along the inhibitory pathway initiated by PGE2 on ERK1/2.

Although the data showed modulation of ERK1/2 activity by PGE2, PI3K, and PKA which are all mediators of the FTY720P-induced stimulation of the ATPase, yet this does not prove that ERK is along the signaling pathway through which FTY720P exerts its stimulatory effect on the Na^+/K^+ ATPase. In fact, upon inhibition of ERK1/2 with PD98059, FTY720P was still capable of inducing ATPase stimulation. PD98059 alone did not have any effect on the ATPase. Had FTY720P been exerting its effect via an inhibition of ERK1/2, then inhibiting ERK1/2 should lead to a similar increase in the ATPase activity which was not the case. It was concluded that ERK1/2 is not a mediator of the signaling pathway.

CHAPTER VI

CONCLUSION

In conclusion, at 2 hours, FTY720P increases the activity of Na^+/K^+ ATPase by binding to and activating S1PR2. This triggers the stimulation of Gq, which in turn, induces the PLC pathway and conventional PKC activation. PKC leads to the phosphorylation, subsequent ubiquitinylation and degradation of IκB thus removing the inhibition from the transcription factor NFκB. Consequently, NFκB translocates to the nucleus and induces the transcription of COX-2 enzyme. COX-2, then, catalyzed the synthesis of PGE2 which is released and binds to EP4 receptors activating Gs proteins. Gs, in turn, activate adenylyl cyclase increasing cAMP production. Elevated cAMP levels stimulate PKA by binding to its regulatory subunits. PKA activates PI3K which leads to a significant increase in Na^+/K^+ ATPase activity. PKA can also inhibit ERK1/2 while PI3K activates it. However, ERK1/2 is not along the stimulatory signaling pathway initiated by FTY720P on Na^+/K^+ ATPase.

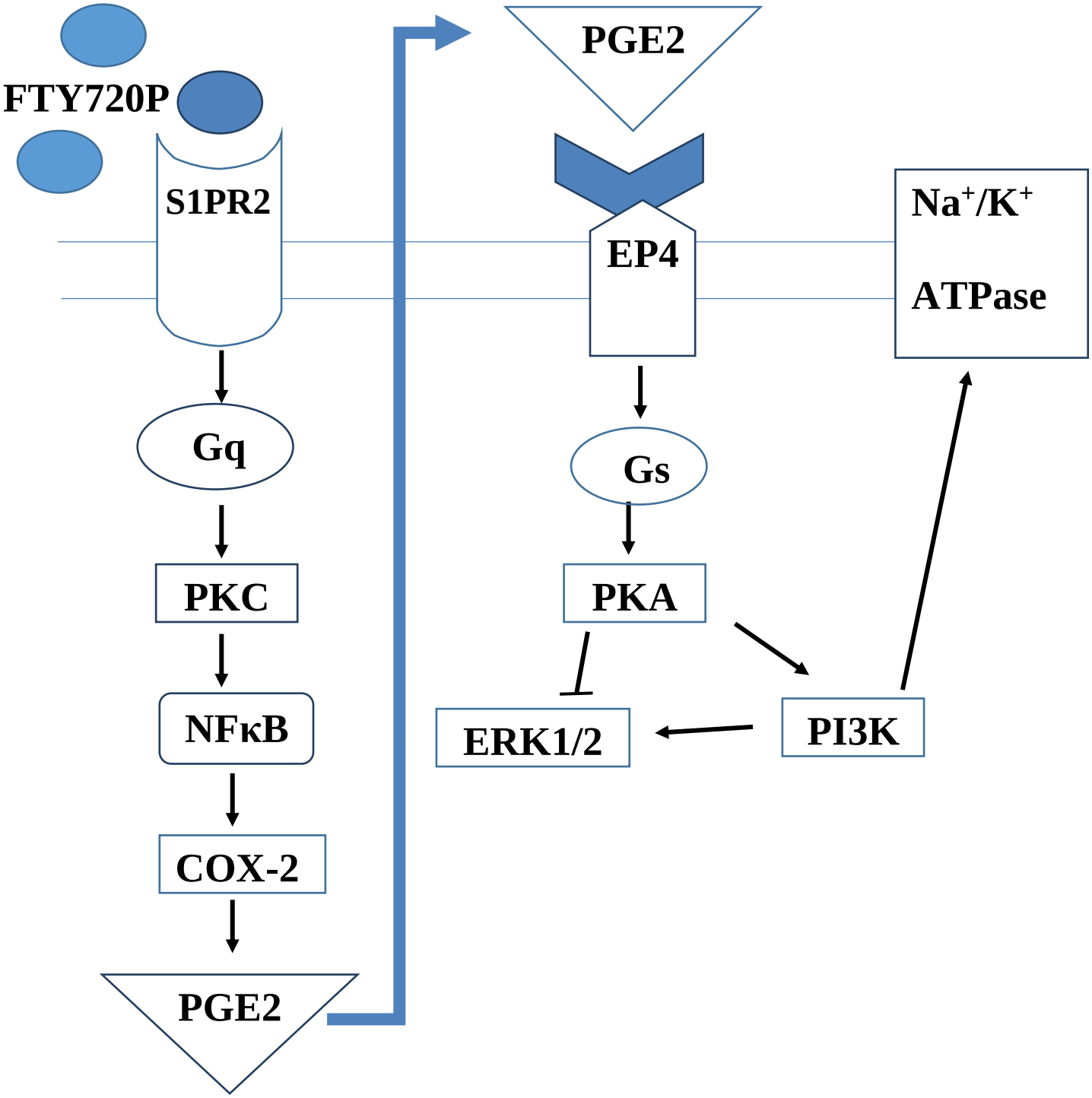

Figure 28: The stimulatory signaling pathway triggered by FTY720P on Na^+/K^+ ATPase at 2 hours

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