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THE EFFECT OF FTY720P ON NA^+/K^+ ATPASE IN CACO2 CELLS, AND THE SIGNALING PATHWAY INVOVLED

by
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AN ABSTRACT OF THE THESIS OF

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The colon plays an important role in sodium absorption which is driven by the sodium electro-chemical gradient established by the Na^+/K^+ ATPase present on the basolateral side. Water follows then by osmosis. Any change in the activity of the ATPase is expected to alter sodium and potassium colonic transport and consequently water, leading to diarrhea or constipation. Diarrhea is the main symptom of Inflammatory Bowel Disease (IBD). IBD patients have lower Na^+/K^+ ATPase activity and higher levels of sphingosine-1-phosphate (S1P), a lipid mediator essential for cell proliferation, migration, and differentiation. S1P acts as an intracellular and an extracellular messenger. Its extracellular action is mediated via 5 different G-protein coupled receptors (S1PR 1-5). Lately, FTY720-P has been recognized as a structural analogue of S1P and has been approved for the treatment of multiple sclerosis.

Since in IBD the high S1P levels are accompanied with low Na^+/K^+ ATPase activity, this work was undertaken to see if a cause effect relationship exists between S1P and the ATPase, and in case it does to determine the signaling pathway involved using Caco-2 cells as a model and FTY720P, a S1P analogue. The activity of the Na^+/K^+ ATPase was assayed by measuring the amount of inorganic phosphate liberated in presence and absence of ouabain, a specific inhibitory of the ATPase. The type of S1P receptors expressed was studied by western blot analysis and showed that S1PR2 is the most abundantly expressed receptor in Caco-2 cells. FTY720P (7.5 nM, 15 min) exerted a significant inhibitory effect on the ATPase which disappeared in presence of JTE-013(a specific blocker of S1PR2) and indomethacin (an inhibitor of cyclooxygenase enzymes), and mimicked by CYM5520, (a S1PR2 agonist). Sulprostone (an EP3 agonist) and RpcAMP (a PKA inhibitor) reduced individually the activity of the Na+/K+ ATPase. The effect of FTY720P was abolished in the presence of calphostin (PKC inhibitor), wortmanin (PI3K inhibitor) and PTIO (nitric oxide scavenger), but the effect of PGE2 disappeared only in presence of PTIO, suggesting that NO is downstream of PGE2 while PKC and PI3K are upstream.The involvement of PKC and NO was confirmed when a similar inhibitory effect to that observed with

FTY720P appeared upon treating the cells with PMA (PKC activator), or SNAP (nitric oxide donor).The effect of PMA was not manifested however, when PI3K was inhibited, inferring that PKC is upstream of PI3K.

It was concluded that FTY720-P inhibits the ATPase by binding to S1PR2 which activates PKC and then PI3K, leading to PGE2 production. PGE2 reduces cAMP levels via its EP3 receptors and increases nitric oxide levels resulting in an inhibitory effect on the Na^+/K^+ ATPase.

CONTENTS

ILLUSTRATIONS

ABBREVIATIONS

AA	Arachidonic acids
ABC	ATP-binding cassette transporters
AC	Adenylyl cyclase
ADP	Adenosine diphosphate
AH	Aqueous humor
Alk-SMase	Alkaline sphingomyelinase
AMPK	AMP-activated protein kinase
AP	Activator protein
AT1	renal angiotensin type 1 receptor
ATCC	American Type Culture Collection
ATP	Adenosine triphosphate
BAD	Bcl-2-associated death promoter
BAEC	bovine aortic endothelial cells
Bcl-2	B-cell lymphoma 2
BH4	Tetrahydrobiopterin
C/EBP	Ccaat- enhancer- binding protein
cAMP	cyclic adenosine monophosphate
CBP	CREB binding protein
CDase	Ceramidase
Cer	Ceramide
CFS-1	Colony-stimulating factor 1

cGMP	Cyclic guanosine monophosphate
CNS	Central Nervous System
COX	Cyclooxygenase
CRC	Colorectal cancer
CREB	cAMP response-element-binding protein
DAG	Diacylglycerol
dbcAMP	2′-O-Dibutryryladenosine 3′,5′-cyclic monophosphate sodium salt
DMEM	Dulbecco's Minimal Essential Medium
DMSO	Dimethyl sulfoxide
EDG	Endothelial differentiation gene receptors
EGFR	Epidermal growth factor receptor
EP	E prostanoid
ER	Endoplasmic reticulum
ERK	Extracellular signal-regulated kinase
FAD	Flavin adenine dinucleotide
FBS	Fetal Bovin Serum
FDA	Food and Drug Administration
FKHR	Forkhead
FMN	Flavin mononucleotide
FOXO1	Forkhead box protein O1
FOXO3a	Forkhead box protein O3
FOXO4	Forkhead box protein O
GAP	GTPase-activating protein
GLUT4	Glucose transporter type 4

GPCR	G-protein coupled receptor
GSK3	Glycogen synthase kinase 3
GTPase	Guanosine triphosphatase
HRP	Horse raddish peroxidase
IBD	Inflammatory Bowel Disease
IFN-γ	Interferon γ
IGF	Insulin-like growth factor
IL-1β	interleukin 1-β
IL-6	Interleukin 6
IP3	Inositol triphosphate
ISP-1	Myriocin
JAKs	Janus kinases
kDa	Kilodalton
MARCKS	Myristoylated alanine-rich C kinase
MS	Multiple Sclerosis
mTOR	Mammalian target of rapamycin
NADPH	Nicotinamide dinucleotide phosphate
NF-κB	Nuclear factor kappa B
NHEs	Na^+/H^+ exchangers
NK	Natural killer cells
NO	Nitric oxide
NOS	Nitric oxide synthase
NPE	Nonpigmented ciliary epithelium
p38-MAPK	p38 Mitogen Activated Protein kinase

PBS	Phosphate Buffered Saline
PDE	Phosphodiesterase
PDGF	Platelet derived growth factor
PDK-1	Phosphoinositide-dependent kinase-1
PG	Prostaglandin
PH	Pleckstrin homology
PI3Ks	Phosphoinositide 3-kinases
PKA	Protein kinase A
PKB	Protein kinase B
PKC	Protein kinase C
PKG	Protein kinase G
PLA2	phospholipase A2
PLC	Phospholipase C
PMA	phorbol 12-myristate 13-acetate
PP	Protein phosphatase
PP2A	Protein phosphatase 2A
PS	Phosphatidylserine
PTEN	phosphoinositide-lipid 3-phosphatase
RGS	Regulator of G-protein signaling
RpcAMP	Adenosine-3′,5′-cyclic Monophosphorothioate Rp-Isomer Triehtylammonium salt
S1P	sphingosine-1-phosphate
S1PR	sphingosine-1-phosphate receptor
S6K	p70 ribosomal S6 kinase
SFK	Src family kinase

sGC	soluble guanylyl cyclase
SGK	Serum- and glucocorticoid-induced kinase
SHIP	SH2- containing inositol (poly)phosphate 5- phosphatase
SK	Sphingosine kinases
SM	Sphingomyelin
SPH	Sphingosine
SPL	S1P lyase
SPNS2	Sphingolipid transporter spinster homolog 2
SPP	S1P phosphatases
SR	Sarcoplasmic reticulum
STAT3	Signal transducer and activator of transcription 3
TKRs	Tyrosine kinase receptors
TNF-α	Tumor necrosis factor α
TXA2	Thromboxane A2
UC	ulcerative colitis
VEGF	Vascular endothelial growth factor
FTY720	Fingolimod

CHAPTER I

INTRODUCTION

Sphingosine-1-phosphate (S1P) is a lipid mediator produced in all body tissues and fluids. It regulates cell proliferation, immune-cell trafficking and migration, differentiation (Smith et al., 2012) and many other processes, and acts via 5 receptors (S1PR1-5) also called endothelial differentiation gene receptors.

Under normal conditions, S1P levels are low in gut epithelial cells, but increase significantly during inflammation. In inflammatory bowel disease (IBD), sphingosine kinases are upregulated leading to higher levels of S1P (Murphy et al., 2011). Diarrhea is one of the main symptoms of IBD and is due to reduced water absorption as a consequence of altered electrolytes movements. The IBD-associated diarrhea was found lately to result from lower NaCl absorption rather than increased chloride secretion (Priyamvada et al., 2015). Sodium absorption from the colon occurs through Na+ channels or through the coupled activity of the Na^+/H^+ and $Cl^-/HCO3^-$ exchangers. In both cases the driving force for the work of the transporters is the sodium gradient established by the Na^+/K^+ ATPase located in the basolateral membrane (Martı´nez-Augustin et al., 2009). A decrease in the activity of Na^+/K^+ ATPase diminishes the sodium gradient, and thus reduces sodium and water absorption leading to diarrhea. Since IBD is accompanied with higher levels of S1P and diarrhea, S1P may be targeting the Na^+/K^+ ATPase in the colon. Such a question has not been addressed before.

Recently, FTY720-P has been recognized as a structural analogue of S1P and has been approved by the FDA for the treatment of multiple sclerosis. In addition it is considered to possess a potential therapeutic use in the treatment of cancer, and showed promising effects in this field. It is not however without any side effects. Administration of the drug was found to cause bradycardia and increased sensitivity to infections (Murphy et al., 2011). Its side effects on colonic activities and ion transport processes geared by Na^+/K^+ ATPase have not been studied yet.

The aim of this work was to

1. Study the effect of FTY720P on the activity of the Na^+/K^+ ATPase in Caco-2 cells (human epithelial colorectal adenocarcinoma cells).
2. Determine the S1P receptors involved.
3. Determine the signaling pathway involved in the effect of FTY720P on the ATPase.

CHAPTER II

LITERATURE REVIEW

A. Na^+/K^+ ATPase

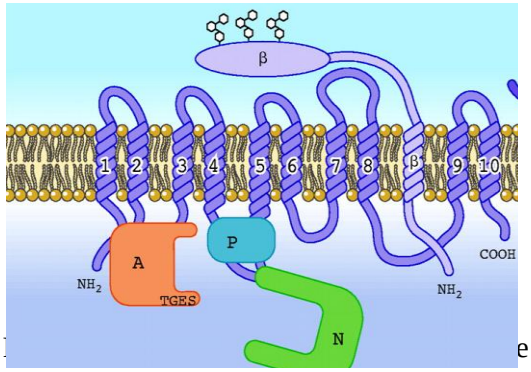
1. Properties

The Na^+/K^+ ATPase or the Na^+/K^+ pump belongs to the P-type ATPase family that couples ion transport to adenosine triphosphate (ATP) hydrolysis. The family is divided into five subfamilies depending on the ligand transported. The Na^+/K^+ ATPase is part of subfamily II which includes in addition, the sarcoplasmic reticulum $Ca2^+$ ATPase, the gastric H^+/K^+ ATPase, and the H^+-ATPase. The Na^+/K^+ ATPase and the H^+/K^+-ATPase are antiporters, while the others, are pumps involved in primary active transport processes (Durlacher et al., 2015).

The Na^+/K^+ ATPase (NKA) consumes about 15% to 20% of the body energy (Suhail et al., 2010). It is expressed in all mammalian cells in a tissue specific manner. The erythrocytes for example have 160,000-fold less Na^+/K^+ ATPase than brain cortex (Bhatia et al., 2016). The Na^+/K^+-ATPase transports three sodium ions outside the cell and two potassium ions into the cell, for every ATP molecule hydrolyzed , maintaining thus a sodium and potassium electro-chemical gradient across the membrane (Suhail et al., 2010). This gradient is used to drive many secondary active transport processes via different transporters such as the Na^+/H^+ and the $Na^+/Ca2^+$ exchangers, and the Na^+-K^+-$2Cl^-$ cotransporter, which regulate respectively cytoplasmic pH, $Ca2^+$ levels, and cellular volume (Bachmann et al, 2011). In addition the pump plays a key role in the generation of action potentials in excitable tissues (Martínez-Augustin et al., 2009).

The Na^+/K^+ ATPase is made up of an α and a β subunits (Bhatia et al., 2016). An additional γ subunit is expressed only in the kidney.

The α subunit is about 110 kDa and has 10 transmembrane helices containing the binding sites for Na^+ (in the intracellular region of the subunit) and K^+ (in the extracellular region of the subunit), and three cytoplasmic domains, the actuator (A), the nucleotide-binding (N), and the phosphorylation (P) domain where ATP hydrolysis takes place. It is the catalytic subunit. It can act also as a signal transducer that regulates the activity of various proteins and kinases (Durlacher et al., 2015). Ankyrin links the catalytic subunit to spectrin cytoskeleton, stabilizing thus the Na^+/K^+ ATPase to the plasma membrane (Bhatia et al., 2016). In humans, there are four α isoforms encoded by different genes. Alpha1 is ubiquitously expressed, α 2 is present in Muscle, heart and brain,α 3 in CNS and brain, and α 4 is expressed in testis (Durlacher et al., 2015).



Horisberger J. D. (2004). Recent Insights into the Structure and Mechanism of the Sodium Pump. Physiology Vol. 19 no. 6, 377-38

The glycosylated β subunit is about 55 kDa with a single transmembrane domain; this subunit is essential for proper pump assembly and targeting to the plasma membrane. It helps also in maintaining polarization of epithelial cells, and prevents motility by dimerizing to a neighboring β subunit forming β - β bridges that enhance adhesion between cells (Durlacher et al., 2015). There are three β isoforms: β1 is ubiquitous, β2 is expressed in skeletal muscle and heart, and β3 is expressed in testis and central nervous system (Suhail et al., 2010).

The γ subunit is about 12 kDa; it is hydrophobic with a single transmembrane domain. Seven γ subunits have been identified; they belong to the FXYD family proteins and modulate the activity of the Na^+/K^+ ATPase (Suhail et al., 2010; Arnaiz and Ordieres et al., 2014).

Having different isoforms of each subunit allows for different combinations and variations in the pump between different tissues (Durlacher et al., 2015).

2. Regulation

Regulation of Na^+/K^+ ATPase activity is essential for the proper functioning of cells, and any alteration may lead to the development of some diseases. Short-term regulation of the ATPase occurs within minutes to hours via mediators such as protein kinase A (PKA), PKC or PKG that phosphorylate serine residues on the alpha subunit or via dephosphorylation by protein phosphatases. Long-term regulation affects transcription and translation of the pump's subunits, and needs longer periods (Köksoy et al., 2007).

Interferon γ (IFN-γ), tumor necrosis factor α (TNF-α), and interleukin 1-β(IL-1β) reduce the expression and activity of the sodium potassium pump. This inhibitory effect of IFN-γ on the pump was observed in colonocytes leading to a decrease in the sodium electro-chemical gradient and Na^+ absorption and an increase intracellular Na^+ (Amasheh et al. 2008; Farkas et al., 2011 & Zeissig et al., 2008), water and consequently, cell volume. IFN-γ reduced also the expression of Na/K/2Cl cotransporter and occludins leading to lower chloride absorption and higher tight junction permeability (Sugi et al., 2001). The effect of IFN- γ on the pump was found to be mediated via an activation of PKC, ERK2, and p38 MAPK (Magro et al., 2004).

The Na^+/K^+ ATPase plays an important role in the regulation of the cardiocytes function. Heart failure was found to be accompanied with a lower activity of the pump and higher levels of TNF- α ; this was shown to act via activation of NF-κB and AP-1 which in turn induce expression of COX-2 and PGE2 production, the latter exerts an inhibitory effect on the pump (Skayian and Kreydiyyeh et al., 2006). Inhibiting the ATPase with cardiac glycosides like ouabain is a well-known therapeutic procedure for heart failure and cardiac arrhythmias (Durlacher et al., 2015).The glycoside has different affinities for the different α subunits, the highest being for α1.

Phosphorylation/dephosphorylation alters the activity of the Na^+/K^+ ATPase. Phosphorylation can be on tyrosine, serine or threonine and can lead to activation or inhibition. PKC phosphorylates Ser11, 16 or 18 of the α subunit resulting in an increase or a decrease in the activity of the pump depending on the cell type (Köksoy et al., 2007) and the PKC isoform: for example, activation of PKC β by renal angiotensin type 1 (AT1) receptors induces phosphorylation of the Na^+/K^+-ATPase α1-subunit and consequently a higher interaction with adaptor protein 1 (AP-1) resulting in the recruitment of the enzyme to the plasma membrane (Efendiev, R. et al., 2000 and Efendiev, R. et al., 2003). On the other hand , phosphorylation of the α1-subunit by PKCζ, when activated by dopamine D1-like receptors in renal proximal tubules, results in binding of PI-3K and adaptor protein 2 (AP-2), promoting endocytosis of the ATPase (Pedemonte et al., 2005; Efendiev et al., 2003&Cinelli et al., 2008).

The *in vitro* activity of purified Na^+/K^+ ATPase was decreased upon phosphorylation by PKA (Bertorello et al., 1991). The effect on the same tissue may vary with the species. While PKA activates the pump in the dog salivary glands, (Komabayashi et al., 1988) it exerts no effect in the rat. The kinase can act directly on

the ATPase or through different intermediates. PKA is inhibited by the S1P receptor that is coupled to Gi/o. Another type of G-protein coupled receptor that affects ATPase activity through PKA is the dopamine receptor. In the striatum, PKA and the ATPase activities change in opposite directions according to the duration of morphine treatment: Short term treatment activates dopamine 2 receptors which are coupled to Gi proteins leading to a stimulation of the Na^+/K^+ ATPase, while long term treatment activates dopamine 1 receptors which are coupled to Gs proteins leading to an inhibition of the ATPase (Pearce et al., 2010).

Phosphorylation and modulation of the ATPase activity can occur also by phophatidyl inositol- 3- kinase (PI3K). Stretching in aortic smooth muscle cells activates PI3K and increases the ATPase activity (SONGU-MIZE et al., 2001). The kinase is involved also in the trafficking of the pump between the cell membrane and intracellular stores. In rat renal proximal tubules, activation of PI3K by dopamine leads to endocytosis of Na^+/K^+ ATPase from the basolateral plasmalemma (Chibalin et al., 1999; Chibalin et al., 1998).

PKC phosphorylation of NH2-terminal tail of the Na^+/K^+ ATPase α-subunit permits PI3K to bind to an adjacent proline-rich sequence (Chibalin et al., 1998), recruiting clathrin adapter complexes (Ogimoto et al., 2000) and internalizing the Na^+/K^+ ATPase. In LLC-PK1cellswhich are renal proximal tubule cells, albumin at physiologic levels was shown to activate PI3K/ PKB pathway leading to PKC activation, PKA inhibition and upregulation of the α1 subunit of the Na^+/K^+ ATPase and an increase in its activity. This pathway is inhibited however, at higher albumin concentration, like what happens in renal diseases (Peruchetti et al., 2001).

Regulation of the pump can occur also via the γ-subunit which is present only in renal epithelial cells (Therien & Blostein et al., 2000; Pu et al., 2001). The subunit decreases the pump affinity to Na+ and K+ (Suhail et al., 2010; Arnaiz and Ordieres et al., 2014), and hence reduces its activity. The γ-subunit is destabilized by phosphorylation by protein kinases A and C removing thus its inhibitory effect on pump (Nelson et al., 1987; Morrow et al., 1989).

Hormones affect also the ATPase activity, and the same hormone can have opposite effects on the pump in different tissues. In chick embryos, the thyroid hormone was shown to inhibit the Na^+/K^+ ATPase via activation of PI3K, PKA and PKC, while in rat alveolar epithelial cells, it increased its activity through PI3K (Therien & Blostein et al., 2000). Insulin also increased the activity of the α1and α2 subunits in skeletal muscle, via phosphorylation of Thr96 by extracellular signal-regulated kinases (ERK) resulting in their translocation and insertion into membrane (Al-Khalili et al., 2004).

Nitric oxide is another important signaling molecule reported to modulate the activity of the Na^+/K^+ ATPase. Nitric oxide (NO) stimulates guanylate cyclase leading to cGMP production and PKG activation. cGMP was shown to exert an inhibitory effect on the Na^+/K^+ ATPase in the colon (Schreiner J. et al., 1980), skeletal muscle (Li & Sperelakis N. et al., 1993), brain (Pontiggia et al., 1998), and cultured alveolar cells (Guo et al., 1998). cGMP/PKG sometimes antagonizes the effect of cAMP/PKA (Carre et al., 1995). The effect of PKG on the pump is isoform specific. In Purkinje neurons, PKG acts on the α3- but not α1-isoform (Nathanson et al., 1995), while in brain endothelial cells it acts on α1- but not α2- or α3-isoform (Pontiggia et al., 1998).

Prostaglandin E2 is a recognized modulator of Na^+/K^+ ATPase activity. In rat hippocampal slices, it inhibited the pump via PKA and PKC activation and increased

phosphorylation of Ser943 of the subunit. A similar inhibition was also observed *in vivo* (Oliveira, M.S. et al., 2009).

B. Sphingosine-1-Phosphate:

Sphingolipids are membrane lipids conserved among all eukaryotes (Coant et al., 2017 &Barros et al., 2014). They are derived from ceramides (Cer) which are synthesized *de novo* by condensation of serine and palmitoyl-CoA in the cytosolic leaflet of the smooth endoplasmic reticulum (ER), and then transferred to the Golgi apparatus where they are converted to sphingomyelin (SM) and incorporated into glycosphingolipids transported later on to the plasma membrane (Coant et al., 2017). Dietary sphingomyelin is hydrolyzed in the intestinal lumen into ceramide by the activity of alkaline sphingomyelinase (alk-SMase) released from the intestinal mucosa. Ceramide is then hydrolyzed by ceramidase (CDase) to sphingosine (SPH) which diffuses to the intestinal cells, and gets phosphorylated by sphingosine kinases (SK) to S1P (Barros et al., 2014) that is composed of a polar head group and one non-polar tails (Kunisawa et al., 2012; Coant et al., 2017). Intestinal tissue contains high levels of sphingolipids and S1P (Kunisawa et al., 2012).

Two isoforms of sphingosine kinase (SK 1 and 2) have been identified in mammals. SK 1 is present in the cytosol but may translocate to the cytoplasmic leaflet of the plasma membrane or intracellular membranes by external stimuli such as TNF-α. SK 2 is present in the cytosol as well as in the nucleus (Tani et al., 2007). Overexpression of SK1 promotes Ras dependent transformation of fibroblasts into fibrosarcoma cells (Pyne et al., 2016). An increase in sphingosine and/or S1P was shown to induce cell division, while an increase in ceramide resulted in apoptosis. In fact, SM and Cer were reported to

inhibit the development of chemically induced colon cancer in animals (Nguyen et al., 2014). An increase in SK1 expression and consequently S1P levels was observed in ulcerative colitis (UC) and colon cancer. S1P activates NF-κB and STAT3 in intestinal epithelial cells promoting tumor formation. In Inflammatory Bowel Diseases (IBD) patients, higher S1P levels were correlated with an increase in the risk of colitis associated cancer (Liang J.et al., 2013) via an amplification loop involving SK1, S1PR1, NF-κB, STAT3 and IL-6 (PyneJ.et al., 2016). NF-κB is also activated in immune cells increasing expression of TNF-α and IL-6, and enhancing inflammation (Nagahashi et al., 2013). Inhibition of SK or S1PRs was able to prevent inflammation in many animal models including asthma, arthritis and IBD (Snider et al., 2012).

S1P lyase (SPL) irreversibly degrades S1P giving ethanolamine phosphate and hexadecenal inside the cells (Barros et al., 2014). Two specific S1P phosphatases 1 and 2 (SPP1 and SPP2) dephosphorylate S1P back to SPH (Kunisawa et al., 2012). S1P degradation may be blocked in colon cancer since the expression of SPL and S1P phosphatase was found to be reduced (Barros et al., 2014).

S1P regulates cell proliferation, migration, immune-cell trafficking and differentiation. It stimulates vascular maturation and angiogenesis during development and lymphocyte recirculation, and has a protective effect in heart, liver, brain, kidney, and lung (Smith et al., 2012). SK activity is declined during ischemia (Levkau et al., 2013). In intestinal epithelial cells, S1P prevents apoptosis via an Akt-dependent pathway (Greenspon et al., 2009). S1P can act as both an extracellular and intracellular mediator. The intracellularly generated S1P can be exported to the outside of the cell via ATP-binding cassette (ABC) transporters including ABCC1, ABCG2 and sphingolipid transporter spinster homolog 2 (SPNS2) (Nguyen et al., 2014), and acts in a paracrine or

autocrine way (Tani et al., 2007; Smith et al., 2012). S1P has 5 different cell receptors (S1PR1 to S1PR5) called the endothelial differentiation gene (EDG) receptors that are differentially expressed across tissues and cell lines (Tani et al., 2007; Smith et al., 2012). S1PRs are coupled to G proteins and expressed only in higher eukaryotes, and not in lower organisms such as plants and yeast, even if these organisms are responsive to S1P (Spiegel et al., 2002). S1PR1 is essential for lymphocyte recirculation from the thymus and lymph node (Tani et al., 2007). S1PR1, 2 and 3 are ubiquitously expressed in mammals, while S1PR4 is restricted to lymphoid tissues and lung, and S1PR5 to brain, skin, and natural killer (NK) cells (Murphy et al., 2011).

S1PR1 is coupled to Gi/o, whereas S1PR2 and S1PR3 are coupled to Gi/o, Gq and G12/13. S1PR4 and S1PR5 couple to Gi/o and G12/13.

Gi/o signaling may activate 1) the small guanosine triphosphatase (GTPase) Ras and the extracellular signal-regulated kinase (ERK); 2) phosphatidylinositol 3-kinase (PI3K); 3) protein kinase C (PKC) /phospholipase C (PLC) leading to higher intracellular calcium levels; 4) adenylyl cyclase (AC) inhibition that reduces cyclic adenosine monophosphate (cAMP).

Gq signals through PLC pathways.

G12/13 signals through small GTPase Rho and the Rho-associated kinase (Brinkmannet al., 2007).

S1P in plasma is attached to lipoproteins (Tani et al., 2007) and is present there at higher concentrations (0.2 to 0.9 μM) than in tissues (0.5 to 75 pmol/mg), but only 1–2 % of the total plasma S1P is biologically active. Plasma S1P levels increase upon inflammation (Smith et al., 2012; Murphy et al., 2011). The total plasma S1P is 20–100-fold higher than the Kd value of its receptors which range between 8–20 nM (Peters et

al., 2007). Cells migrate towards high concentrations of S1P, and a disruption of this gradient was demonstrated in inflammatory and/or autoimmune disorders including asthma, rheumatoid arthritis (Murphy et al., 2011), type 1 diabetes, and multiple sclerosis (Kunisawa et al., 2012).

Vascular endothelial growth factor (VEGF), platelet derived growth factor (PDGF), insulin-like growth factor (IGF), TNF-α, and IL- 1β activate SK1 by inducing its phosphorylation and translocation from the cytosol to the plasma membrane where sphingosine is localized. SK2 is found in several intracellular compartments including mitochondria and nucleus. S1P in the nucleus inhibits histone deacetylase (Nagahashi et al., 2013 and Smith et al., 2012).

S1P affects endothelial cells by activating endothelial nitric oxide synthase through a G-protein coupled receptor, inducing vasodilation. On the other hand, S1P is able to increase $Ca2^{+}$ concentration and to activate Rho kinase in smooth muscle cells leading to constriction (Peters et al. 2007).

S1P was shown to mediate the effect of different growth factors, hormones and cytokines by enhancing COX-2 expression, which is usually very low under normal physiological conditions, and inducing PGE2 production (Cheng et al. 2016). COX-2 is regulated at the transcription and translation levels (Pettus et al., 2005).The involvement of PGE2 and PGF2α in labor and uterine contractions was ascribed to the presence of sphingosine-1-phosphate in the amniotic fluid that modulates prostaglandin synthesis (Kim et al., 2003).

C. FTY720:

FTY720 (Fingolimod) is derived from the fungal metabolite myriocin (ISP-1) isolated from the fungus *Isaria sinclairii*. FTY720 is lipophilic having a structure similar to sphingosine (Murphy et al., 2011); once inside the cell, it is phosphorylated by SK2 and to a lesser extent by SK1 to generate FTY720-phosphate (FTY720-P). The membrane is not permeable to FTY 720-P because of its amphiphilic nature; consequently it is exported outside the cell by ABC transporters (Ottenlinger et al., 2016).

Non-phosphorylated FTY720 alter the activity of many enzymes. Its anti-tumor effects are due to its inhibition of the tumor suppressor Protein phosphatase 2A (PP2A) by the oncoprotein SET. Activating PP2A enhances necrosis, apoptosis, and cell death. FTY720 can cause apoptosis at micromolar concentrations in human fibroblasts (Potteck et al., 2010). FTY720 also inhibits the cytosolic phospholipase A2 (cPLA2), which is the enzyme involved in eicosanoids biosynthesis, reducing thus the antigen induced secretion of PGD2 in mast cells (Ottenlinger et al., 2016).

FTY720-P acts as an analogue of S1P. It binds to the different S1P receptors and activates their signaling pathways. When bound to S1PR3 receptors, it acts via the Gi proteins only, although the Gq proteins are also coupled to the receptor. Thus FTY720-P does not mimic exactly the effects of S1P on S1PR3 (Christian et al., 2008). However, FTY720-P can signal via the G12/13/Rho/ROCK pathway in myofibroblasts, similarly to S1P, causing myofibroblast contraction (Sobel et al., 2015).

S1PR1 activation by S1P or FTY720P results in receptor desensitization and internalization by clathrin-mediated endocytic pathway (ReevesP.et al., 2016). β-arrestins bind to the activated receptor and target it to clathrin coated pits (Luttrell & Lefkowitz, 2002). Maximal internalization of S1PR1 can take up to 30 min. Recycling

of S1PR1to the plasma membrane occurs if it is induced by S1P but not if induced by FTY720P (Sykes et al., 2014). So FTY720-P may exert opposite effects to those of S1P leading to S1PR1 irreversible internalization and degradation.

FTY720-P has a slow dissociation rate from S1PR1 and thus it is internalized with the receptor, maintaining β-arrestin recruitment and preventing receptor recycling. In contrast, FTY720-P has a high dissociation rate from S1PR3 and consequently is not internalized together with the receptor, lowering the levels of β-arrestin recruited and resulting in S1PR3 recycling back to the cell surface (Ottenlinger et al., 2016). FTY720 was reported to induce downregulation and degradation of S1PR1 in lymphocytes, but had no effects on other S1PRs (Maceyka et al., 2012). S1P, on the other hand, remains attached to S1PR1 and S1PR3 receptors for long periods, so it is likely to be internalized with them, but because of its metabolic instability, it is degraded, β-arrestin is no more recruited, and the receptors are recycled back to the cell surface (Ottenlingeret al., 2016).

FTY720-P is less metabolized (Sykes et al., 2014) than S1P. When CHO cells transfected with the cDNA encoding the human S1PR1 and S1PR3 were incubated with S1P, S1P concentrations were reduced by approximately 30-fold after 3 hrs, while incubation with FTY720-P did not result in any change in concentration over the 3□h incubation period.

Whether FTY720P exerts similar or opposite effects to those of S1P, depends on the molecules in lipid rafts to which the receptor is associated, such as the GPCR-desensitizing arrestins, and the regulator of G-protein signaling (RGS) proteins. In the cardiovascular system, at least ten different RGS proteins are expressed: RGS1 and RGS3 inhibit S1PR1, S1PR2 and S1PR3 signaling, while RGS2 and RGS4 reduce

signaling through S1PR2 and S1PR3 receptors, respectively. The differential expression of RGS between different tissues may regulate cellular responses to S1P and FTY720 (Brinkmann, 2007).

FTY720 plays an important role in inflammation and functioning of immune cells.

The internalization of S1PR1 by FTY720-P leads to inhibition of T-cell egress from secondary lymphoid organs and a decrease in the level of peripheral lymphocytes (Kunisawa & Kiyono, 2012). Also FTY720P prevents immune cells migration to inflammatory sites, making it useful in the treatment of inflammatory disorders. In addition, FTY720 can cross the blood - brain barrier and lead to effective communication between astrocytes and neurons. It was recently approved by the US Food and Drug Administration (FDA) as a first-line treatment for relapsing forms of Multiple Sclerosis (MS) (Brinkmann, 2007).

FTY720 was shown to prevent the rejection and destruction of kidney transplant by inhibiting alloantigen reactive T-cells re-circulation to the grafted tissue probably by acting via the immunomodulatory receptor S1PR1. In overtly diabetic NOD mice, continuous oral FTY720 treatment causes a complete reversal of diabetes in 50% of the animals, even if they still contain diabetogenic cells, but not dominant immunoregulatory cells (Brinkmann, 2007). FTY720 improved chemically induced colitis making it a potential candidate for IBD treatment (Murphy et al., 2011).

FTY720P may have an important therapeutic role in cancer therapy. It inhibits tumor angiogenesis by blocking S1P-mediated Ca^{2+} mobilization which is needed for the migration of vascular endothelial cells. IBD can develop into colorectal cancer (CRC) by an amplification of the NF- κB/IL6/STAT3 loop, a process that can be

eliminated by FTY720-P through a decrease in the expression of SK1, S1P and S1PR1 activation. Even after CRC establishment, FTY720 was able to block tumor progression (Liang et al., 2013), and inhibited tumor growth, angiogenesis and metastasis in various carcinoma models (Meeteren et al., 2008) at doses that are at least 10-fold higher than the dose required for immunomodulation.

S1P slows down pacemaker activity of sinoatrial node cells in vitro, while FTY720 induces a transient asymptomatic reduction in heart rate (Peters et al., 2007). Undesired effects of FTY720P include bradycardia that is induced by S1PR3 activation, higher risks for infections, mainly lower respiratory tract infections (Christian et al., 2008). The need for compounds with the same benefits of FTY720P but less side effects requires information about its receptors binding and downstream signaling.

D. Protein kinase C:

Protein kinase C (PKC) is a family of serine /threonine enzymes that alter the activity of other proteins by phosphorylating their hydroxyl groups. PKCs are involved in many cellular activities like desensitizing receptors, controlling gene transcription, mediating immune responses, and regulating cell growth (Asaoka et al., 1992). In quiescent cells, 90% of PKCs are inactive (Mari et al., 2005). In the colon, PKC plays an important role in inflammation, ion transport, protein secretion, and carcinogenesis of the gastrointestinal tract (Mari et al., 2005). PKCs affect barrier formation and function by regulating phosphorylation and localization of tight junction proteins like the translocation of claudin-1 and ZO-2 from cytosol to membrane.

PKC is activated by an increase in cytosolic calcium or diacylglycerol (DAG) which result from the cleavage of phospholipids by activated membrane-bound

phospholipase C (PLC).The activated kinase regulates many enzymes, including the Na^+/K^+ ATPase, leading to its stimulation or inhibition (Castagna et al., 1882).

Protein kinase C is a single polypeptide, consisting of four domains C1–C4 (Coussens, 1886). Starting from the N-terminal end, the C1 domain contains a Cys-rich motif, duplicated in most isozymes; it is the diacylglycerol/ phorbol ester binding site (Bell, 1991). The C2 domain binds acidic lipids and Ca2+ in some isoenzymes (Newton, 1995). The C3 and C4 domains form respectively the ATP- and substrate-binding site for the kinase activity (Taylor &Radzio-Andzelm, 1994). An autoinhibitory pseudosubstrate sequence precedes C1 (House & Kemp, 1987).

There are 11 protein kinase C isozymes classified, according to their structure and cofactor regulation, into three groups (Nishizuka, 1995). 1) The conventional protein kinase Cs: α, βI, βII and γ, distinguished from other groups in possessing a Ca2+ binding site in the C2 domain and in being regulated by Ca2+; 2) The novel protein kinase Cs: PKCδ, PKCε, PKCη (L), PKCθ and PKCμ: They are structurally similar to the conventional protein kinase Cs, except that the C2 domain, does not bind Ca2+. 3) The atypical protein kinase Cs: ζ and λ: They differ in structure from the other two classes; C1 domain contains only one Cys-rich motif (not two), and key residues that maintain the C2 fold do not appear to be present. Furthermore, isozymes in this group have been reported not to respond to phorbol esters *in vivo* or *in vitro* (Nishizuka, 1995).

Protein kinase C phosphorylates serine or threonine present in a specific sequence, and possesses an ATPase activity that catalyzes the hydrolysis of ATP (O'Brian & Ward, 1991), and a phosphatase activity in presence of excess ADP (Asaoka et al., 1992).

Protein kinase C is regulated by two distinct mechanisms: the first is maturation induced by phosphorylation, which aligns residues for catalysis and localizes protein kinase C to the cytosol, and the second is binding of cofactors that activates the enzyme and promotes its membrane association and pseudosubstrate removal.

To become catalytically competent and mature, the enzyme should undergo three phosphorylations: Phosphorylation by phosphoinositide-dependent kinase-1 (PDK-1) at C4 domain, followed by 2 autophosphorylations in the catalytic domain (Keranen et al., 1995; Tsutakawa et al., 1995). Phosphorylation changes PKC conformation into a closed one whereby the pseudosubstrate occupies the active site, this is why the mature protein kinase C remains in the cytosol (Keranen et al., 1995) and inactive (Griner et al., 2007).

Translocation to the membrane requires binding of cofactors. Ca2+ binds to C2 domain of mature PKC (Orr & Newton 1992; Webb, 2000) and increases the affinity of conventional protein kinase Cs for acidic and negatively charged lipids (Bazzi & Nelsestuen, 1987), by changing the conformation of the lipid-binding surface. This does not apply to novel protein kinase Cs, whose lipid binding surface doesn't need further modulation. In resting conditions, Ca2+ levels are not enough to activate PKC. For conventional and novel PKC, DAG is essential for activation and membrane penetration (Webb, 2000). DAG and phorbol esters bind to C1 domain, increase the affinity of PKC to the membrane by capping the hydrophilic ligand groove, decreasing surface hydrophobicity (Zhang, 1995). They induce also conformational changes that lead to the release of the pseudosubstrate freeing thus the enzyme from its inhibitory effect, and allowing substrate binding and phosphorylation. Novel PKCs travel to membranes more slowly because they don't bind Ca^{2+} (Griner et al., 2007). Phosphatidylserine (PS) binds

to the C2 domain, which together with DAG, increase the affinity of PKC binding to the membrane, resulting in pseudosubstrate release and maximal activation. Also the presence of diacylglycerol causes a selective increase in conventional and novel protein kinase C's affinity for phosphatidylserine (Newton, 1993). Atypical PKC is activated by lipid components, such as phosphatidylinositol, phosphatidic acid, arachidonic acid, and ceramide (Xiao & Liu, 2013).

The pseudosubstrate is removed in all three groups of protein kinase C: conventional (activated by phosphatidylserine, diacylglycerol, and Ca2+ binding), non-conventional (activated by short chained phosphatidylcholines binding (Walker & Sando, 1988)), or atypical (activated by protamine binding) (Orr & Newton, 1994; Takai, 1979).

Translocation to the membrane has been conventionally used as a hallmark for PKC activation.

PKC isozymes are activated by various agents, including hormones, growth factors, and other membrane receptor ligands. PKC isozymes regulate diverse cell functions directly or indirectly, including cycle regulation (e.g., MARCKS, p53, and p21), cell adhesion (e.g., adducins and integrins), DNA synthesis and transcription (e.g., transcription factor C/EBP and glycogen synthetase kinase 3β), cell motility (e.g., RhoA and integrins), apoptosis (e.g., Bad and Bcl-2), drug resistance (e.g., P-glycoprotein), and cell growth and differentiation (e.g., induced by epidermal growth factor receptor (EGFR)) (Kang, 2014).

In cancer, PKC isozymes stimulate survival signaling pathways, such as Ras/Raf/MEK/ERK or PI3K/Akt (also known as PKB)/mTOR pathways, but suppress the expression of cancer suppressor-associated or apoptotic signals such as the caspase

cascade or the Bax subfamily proteins (Kang, 2014). In colon cancer, PKC-α is downregulated, but decreases proliferation via downregulation of the Wnt/β-catenin pathway (Gwak et al., 2009).

PKC can regulate ion channels and transporters. In epithelial monolayers, nPKCε was shown to reduce chloride secretion by increasing endocytosis of the Na+-K+-2Cl+ transporters (Song et al., 1999). Increased endocytosis by nPKCε is opposed by cPKCα (Song et al., 2002). In CaCo-2 cells, phorbol 12-myristate 13-acetate (PMA), a PKC activator, decreased the activity of the apical Cl^-/ $HCO3^-$ exchanger via activation of phosphatidylinositol 3-kinase. The Cl^-/$HCO3^-$ and Na+/H+ exchangers are coupled in the mammalian colon (Saksena et al., 2002; Powell 1987). Na^+/H^+ exchangers (NHEs) mediate the exchange of extracellular Na^+ with intracellular H^+; In CaCo-2 cells, cPKCα activation reduced apical Na^+ transport, and inhibited NHE3 transcription (Alrefai et al., 2001).

E. Phosphoinositide 3-kinases:

Phosphoinositide 3-kinases (PI3Ks, PtdIns 3-kinase), are activated by specific Tyr kinase receptors (TKRs) or GPCRs. They phosphorylate the 3-OH group of inositol ring of membrane lipids, producing PtdIns(3)*P*, PtdIns(3,4)*P*2 and PtdIns(3,4,5). These lipids attracts pleckstrin homology (PH) domain containing proteins, and recruits them to the cell membrane (Vanhaesebroeck et al., 2001). They activate a variety of intracellular proteins involved in cell mitogenesis (Fantlet al., 1992), glucose uptake (Hara et al., 1994*)*, actin rearrangements (Kotaniet al., 1994), chemotaxis, and secretion (Wennstromet al., 1994). In the basal state, PtdIns(3)P is present at higher levels than

PtdIns(3,4)P2 and PtdIns(3,4,5)P3 (Traynor-Kaplan et al., 1989). PI3K catalytic subunits have serine/threonine kinase activity (Vanhaesebroeck et al., 1999).

PI3Ks are made of a p110 catalytic subunit and a p85 regulatory adapter subunit. They are classified into three classes based on their substrate preferences and sequence homology (Vanhaesbroeck et al., 1997). Class I PI3Ks phosphorylate PtdIns(4,5)*P*2 producing PtdIns(3,4,5)*P*3 (Hawkins et al.,1992), which regulates glucose homeostasis, cell migration, growth, and proliferation in mammals (Engelman et al., 2006). Class II PI3Ks are large proteins with their catalytic domain having 45 to 50% homology to class I PI3Ks; they phosphorylate PtdIns and PtdIns-4-P *in vitro*. Class III PI3Ks phosphorylate only PtdIns to produce PtdIns(3)*P*, and they control vesicle transport (Rameh et al., 1997). Mammals have eight isoforms of PI3K: four isoforms belong to class I, three are part of class II and one is in class III (Sjolander et al., 1991).

The p110 subunit has four isoforms α, β, γ and δ. The p85 subunits are encoded by three mammalian genes p85α, p85β and p55γ. In fibroblasts, the p85 adapter subunit recruits the p110 catalytic subunit to an activated receptor tyrosine kinase (Kazlauskas, 1994) inducing its activation and phosphorylation of PtdIns(4,5)*P*2 to PtdIns(3,4,5)*P*3. Chemokine receptors control heterotrimeric G proteins that activate p110γ (Stephens et al., 1994). The catalytic subunits p110 can also bind to the active conformation of Ras, which might stabilize p110 association with the plasma membrane and its activation after its recruitment to a receptor complex (Rodriguez-Viciana et al., 1994). p85 not only translocates catalytic subunit but also negatively regulates the catalytic activity of its associated p110 subunit (Kodaki et al., 1994).

PI3K may also be activated in a receptor-independent manner; for example in leucocytes and during cytokine signaling, it is activated by non-receptor tyrosine kinases such as the Janus kinases (JAKs). Haematopoietic cytokines (such as interleukin 3 and 2), activate Shc, Grb2 and Gab2 adapters that form a signaling scaffold to translocate PI3K (Gadina et al., 2000).

PtdIns (3, 4, 5) *P3* is dephosphorylated by the phosphoinositide-lipid 3-phosphatase (PTEN) (Cantley and Neel, 1999) and by the SH2- containing inositol (poly) phosphate 5- phosphatase SHIP (Rohrschneider et al., 2000), to generate PtdIns(3,4)*P*2 that binds to PH domains of proteins, modifying their activity or inducing their re-localization (Rameh et al., 1997). PTEN is a tumor suppressor and cells lacking PTEN exhibit constitutive activation of PI3K signaling pathways. SHIP loss results in an unbalanced immune response, and the development of autoimmunity (Helgason et al., 1998).

PI3K activates the Tec family of tyrosine kinases (Shan et al., 2000), the serine/threonine kinase Akt/PKB (Cantley& Neel, 1999) and Rac/Rho GTPases (Liliental et al., 2000). In addition, PI3Ks activate phosphoinositide-dependent kinase 1 (PDK1) that controls many kinases involved in distinct cellular processes. PDK1 has an N-terminal catalytic domain and a C-terminal PH domain that binds to PtdIns(3,4,5)*P*3 with high affinity (Anderson et al.,1998). PDK1 phosphorylates AGC protein kinases, protein kinase B (PKB, or Akt), serum- and glucocorticoid-induced kinase (SGK), atypical isoforms of protein kinase C (PKC) (Mora et al., 2004), and p70 ribosomal S6 kinase (p70S6K1), a kinase that is important for the regulation of protein synthesis and cell growth (Alessi et al., 1997).

Protein kinase B (PKB, also known as Akt) is a serine/threonine kinase that mediates PI3K action (Burgering & Coffer, 1995). It is phosphorylated, in addition to PDK1, by the mammalian target of rapamycin complex 2 (mTORC2) (Sarbassovet al., 2005). Akt regulates cell growth, size and survival (Datta et al., 1997). PtdIns(3,4,)*P*2 and PtdIns(3,4,5)*P*3 bind to the PH domain of PKB, recruiting the kinase to the plasma membrane (Astoul et al., 1999). PKB directly phosphorylates pro-apoptotic forkhead (FKHR) transcription factors families, FKHR (FOXO1), FKHRL1 (FOXO3a), and FOXO4 (Manning and Cantley, 2007). Some of the Akt substrates identified are glycogen synthase kinase 3 (GSK3) (Cross, 1995), the pro-apoptotic protein BCL-2 antagonist of cell death (BAD) (Dattaet al., 1997), the cell cycle regulators p21 (Zhou et al., 2001), p27, and AS160 which is a RAB GTPase-activating protein (GAP) that regulates insulin-stimulated exocytosis of glucose transporter type 4 (GLUT4) (Kane et al., 2002).

PI3K isoforms are co-expressed in cells showing similar specificities to lipids substrate. Mice lacking p110γ are viable but have defective neutrophil and lymphocyte function (Li et al., 2000), while mice lacking p85α are not viable and their B cells are functionally deficient (Fruman et al., 1999). PI3K isoforms play different roles in macrophages: From the different p110 isoforms, only p110α (but not p110β or p110δ) is required for mitogenic responses triggered by colony-stimulating factor 1 (CFS-1). In contrast, p110β and p110δ are necessary for CSF-1-induced modulation of actin dynamics. p110γ is activated by G-protein coupled receptors, whereas p110α, p110β and p110δ are activated by tyrosine kinases. In addition, p110γ and p110α/δ operate at different times during a cell lifetime.

F. Nitric oxide:

Nitric oxide is an important signaling molecule involved in an array of physiological and pathophysiological processes. While, at low levels, it is needed for the regulation of many cellular functions, it can turn at high levels, into a cytotoxic agent responsible for many diseases. It is synthesized by the enzyme nitric oxide synthase (NOS) which oxidizes L-Arg to L-citrulline and has oxygen and NADPH as a co substrates, and flavin-adenine dinucleotide, flavin mononucleotide, tetrahydrobiopterin (BH4), and a heme complex (iron protoporphyrin IX) as co-factors.

Three isoforms of the NOS have been identified: neuronal NOS, inducible NOS and endothelial NOS, referred to as NOSI, NOSII, and NOSIII, respectively. The neuronal enzyme (NOS-1) and the endothelial isoform (NOS-3) are calcium-dependent, constitutively expressed and produce low levels of NO. The inducible isoform (NOS-2) is expressed upon stimulation by some factors, is calcium independent and produces large amounts of NO which can be cytotoxic.

The NOS enzyme is a homodimer made of two major domains: a C-terminal reductase domain containing binding sites for FAD, FMN and NADPH, and an N-terminal oxygenase domain containing the binding sites for heme, BH4 and L-arginine. The two domains are linked by a calmodulin -recognition site. The monomeric form of the enzyme is inactive. The heme domain allows for dimerization and activation of NOS (Marletta, 2001; Bredt, 1999). The activity of the constitutive enzymes is controlled by binding of calmodulin triggered by elevation in calcium, while in iNOS, calmodulin is bound permanently and thus not involved in the regulation process.

Endothelial NOS (eNOS) produces NO which induces vascular smooth muscle relaxation and platelet inhibition (Ignarro, 1990).

Inducible NOS (iNOS) is activated in macrophages, neutrophils, endothelial cells and other cell types (Moncada et al., 1991) in response to bacterial endotoxin or cytokines (interleukin-1 or interferon-gamma) (Stuehr, 1987). The produced NO was reported to decrease vascular tone in septic patients (Bryan et al., 2009).

Neuronal NOS (nNOS) produces NO in the central nervous system, which acts as a neurotransmitter that enhances several neural processes including memory (Gillespie et al., 1990)

Nitric oxide diffuses freely across the cell membrane because of its small size and because it carries no charge. NO acts via two mechanisms: cGMP-dependent and cGMP-independent.

In the cGMP-dependent pathway, NO binds to the soluble guanylyl cyclase (sGC) which is a heme-containing protein made up of two subunits α and β; each subunit has two isoforms α1, α2, β1 and β2, but only α1/β1 and α2/β1 heterodimers are activated by NO (Russwurmrt et al., 1998). The α1/β1 isoform is the most abundant and is present at high levels in the brain, lungs, heart, kidneys, spleen and muscles (Budworth et al., 1999). sGS leads to a rise in cGMP concentration which acts directly on downstream effectors such as the family of cGMP-dependent protein kinases (PKGs). In smooth muscle cells, PKG phosphorylates many target proteins resulting in a decrease in the level of intracellular calcium and muscle relaxation. One substrate of PKG is the protein phospholamban that regulates the activity of the sarcoplasmic reticulum Ca^{++} ATPase. Phosphorylation of phospholamban increases the activity of the Ca^{++} ATPase and increases calcium uptake by the sarcoplasmic reticulum (SR) leading to a decrease in intracellular calcium. Another target of PKG is the IP3 receptor located in the SR which is also a calcium channel. Phosphorylation of IP3 receptors decreases

the activity of the channel and prevents the release of calcium from the SR, leading to a decrease in intracellular calcium. PKG has been reported also to phosphorylate calcium channels present in the plasma membrane reducing thus the influx of calcium to the inside of the cell (Carvajal et al., 2000).

Cyclic GMP may in addition, activate cyclic nucleotide-gated channels which are non-specific cation channels found mostly in the retina and olfactory epithelium and involved in visual phototransduction and olfaction, respectively. Cyclic GMP activates in addition cGMP-regulated phosphodiesterases which catalyze the hydrolysis of cAMP and cGMP to the inactive metabolites AMP and GMP. Although many isoforms of PDE exist, only PDE5 catalyses the breakdown of cGMP (Conti, 2000; Denninger & Marletta, 1999). The cGMP/protein kinase G (PKG)-mediated pathway was reported to be involved in the inhibition of the Na^+/K^+-ATPase in aqueous humor (AH)-secreting cells. A similar inhibitory effect of the ATPase was observed in nonpigmented ciliary epithelium (NPE) which was mediated through PKG-dependent Src family kinase (SFK) activation (Shahidullah et al., 2014).

The cGMP-independent pathway affects various cellular processes including mitochondrial cytochrome oxidase inhibition (Giulivi, 1998), calcium-dependent potassium channels activation (Bolotina et al., 1994), NF-κB activation (Sekkai et al., 1998), G-proteins' activation (such as protooncogene p21ras) (Lander et al., 1993), caspase inhibition (a cysteine protease involved in apoptosis) (Li et al., 1997) and cardiac calcium release channels inhibition (the ryanodine receptor) (Stamler, 1997). NO is oxidized to NO_2^- and NO_3^- under physiological conditions, and at high concentrations, it reacts with O2 to form N2O3. N2O3 is a nitrosating agent that reacts with nucleophiles such as the sulfur of cysteine residues. NO has a short half-life since

it is a free radical and reacts with intracellular constituents such as superoxide (Beckman et al., 1993) resulting in the formation of the cytotoxic peroxynitrite anion ($ONOO^-$) (Lipton et al., 1993). This is why excessive generation of NO and $ONOO^-$ plays a role in the pathophysiology of neurological conditions such as stroke, Alzheimer and Parkinson disease (Delwinga et al., 2008).

NO increases the activity of COX-1 leading to a sevenfold increase in PGE2 formation (Salvemini et al., 1993). It can increase also the activity of COX-2, the inducible form of the enzyme. In endotoxin- stimulated mouse macrophage cell line RAW-264, iNOS and COX-2 enzymes were shown to be stimulated resulting in the production of large amounts of NO and prostaglandins (PGs) (Miyamoto et al., 1976). Inhibiting iNOS decreased NO and PG release from these cells (Salvemini et al., 1993), showing that NO exerted a stimulatory action on COX-2 and this action was independent of sGC (Cuzzocrea & Salvemini, 2007).

Endothelial NOS is activated by phosphorylation at Ser-1177, but inhibited by phosphorylation at Thr-495 which is located in the calmodulin binding sequence (Chen, 1999), thus reducing the enzyme affinity to calmodulin. Multiple protein kinases including Akt/PKB, cAMP-dependent protein kinase (PKA), and the AMP-activated protein kinase (AMPK) activate eNOS by phosphorylating Ser- 1177. In endothelial cells, PKC inhibits eNOS activity by phosphorylating Thr-495, and amplifies the inhibition by dephosphorylating Ser- 1177 indirectly through protein phosphatase 2A (PP2A). PKA activates NOS by directly phosphorylating Ser-1177, and induces dephosphorylation of Thr-495 through the action of PP1, serine/threonine phosphatase.

VEGF stimulates at least two protein kinases (Akt and PKC) to control eNOS activity; VEGF-induced phosphorylation of Ser-1177 by Akt is reduced by PKC

signaling (Michell et al., 2001). In bovine aortic endothelial cells (BAEC), when S1P bind to its G protein-coupled EDG receptors, it activates the protein kinase Akt which phosphorylates in turn Ser-1177 residue of eNOS enzyme, increasing NOS activity (Igarashi et al., 2001).

G-Prostaglandin E2:

Prostaglandins (PGs) are active lipid compounds with a variety of physiological and pathological functions, found in all tissues and synthesized from arachidonic acids (AA) liberated from membrane phospholipids by the action of phospholipase A2 (PLA2) (Murakami & Kudo, 2002). AA is converted into unstable endoperoxide intermediates, PGG2 and PGH2 (Hamberg et al., 1974) by the action of cyclooxygenase (COX). The oxygenated intermediate PGH2 generates prostaglandin E2 (PGE2), prostaglandin D2 (PGD2), prostaglandin I2 (PGI2), prostaglandin F2α (PGF2α) and thromboxane A2 (TXA2) (Vaneet al., 1998). Then the prostaglandins are released outside the cell by specific transporters that belong to the ATP-binding cassette family and can interact with specific receptors in an autocrine or paracrine manner (Narumiya, 1994).

There are three isoforms of COX enzyme: COX-1 which is constitutively expressed, COX-2 which is inducible by various external stimuli such as different growth factors, hormones and cytokines (Smith et al., 1994) and COX-3 which is a splice variant of COX-1 and is mostly expressed in the brain and the heart (Chandrasekharan et al., 2002). COX-2 which under normal physiological conditions has very low expression levels (Cheng et al., 2016) is known to be induced by S1P.

From the different prostaglandins, PGE2 is probably the one exerting the widest biological effects. It is involved in inflammatory bowel disease (IBD) (Ahrenstedt et al., 1994), entero-invasive bacterial diseases (Reseta & Barrett, 2002) and colorectal cancers (Eberhart et al., 1994). PGE2 binds to four different types of receptors present in the plasma membrane and known as E prostanoid (EP) receptors EP1, EP2, EP3 and EP4; EP3 and EP4 exhibit also nuclear membrane localization (Bhattacharya et al., 1998, 1999). EP receptors have seven transmembrane domains that are associated to G proteins (Breyer et al., 2001), and are conserved from mouse to human (Bhattacharya et al., 1998).

EP receptors transduce different signals according to the type of G-protein to which they are coupled. EP1 has the lowest affinity for PGE2. In the large intestine, EP1 receptor expression was seen in goblet cells and in other epithelial cell types (Northey et al., 2000). EP1 receptors mediate signaling events by activation of phospholipase C and elevation of inositol triphosphate, diacylglycerol and Ca^{2+}. Consequently, EP1 activates protein kinase C and c-Src. The latter was shown to mediate the transactivation of epidermal growth factor receptor through activation of protein kinase B (Akt) that promotes cell proliferation and invasion (Han & Wu, 2005).

In human colon, EP2 receptors are expressed at the apex of the colonic mucosa while EP4 receptors are expressed in the lateral crypt epithelia (Takafuji et al., 2000) and lamina propria T lymphocytes (Cosme et al., 2000).The EP2 and EP4 receptors stimulate cAMP/protein kinase A (PKA) signaling by activating Gαs and adenylate cyclase. PKA phosphorylates and activates Akt kinase, which indirectly inhibits GSK-3. GSK-3 Inhibition activates and translocates cytosolic β-catenin to the nucleus resulting in cellular proliferation (Cadigan & Nusse, 1997). Although EP2 and EP4 receptors

have common signaling intermediates, yet they have specific and different signaling pathways. EP4 receptor activation in HEK cells stably expressing both EP4 and EP2 receptors induces the phosphorylation of extracellular signal-regulated kinases (ERKs) by a PI3K-dependent mechanism as well as the expression of early growth response factor-1 (Fujino et al., 2003). While EP2 receptors decrease the inhibitory tyrosine phosphorylation of PTEN (a phosphatase that can act as a PI3K pathway inhibitor) rendering PTEN active (White et al., 2005). EP2 activation was shown also to induce transactivation of the EGF receptor leading to increased migration and invasion of colon cancer cells (Pai et al., 2002).

In humans, EP3 receptor is expressed in the apex of colonic mucosa (Takafuji et al., 2000).They may be coupled to Gi or Gq proteins. EP3 receptor were reported also to activate the Ras signaling pathway in lung adenocarcinoma cells (Yano et al., 2002) and activate the small G protein Rho and its target p160 Rho-A binding kinase ROK causing neurite retraction (Katoh et al., 1998). PGE2 plays an important role in gut inflammation. In ulcerative colitis, EP2 and EP3 receptors are overexpressed in intestinal epithelial cells (Takafuji et al., 2000). However, the proinflammatory role of PGE2 is mediated by the high-affinity colonic EP4 receptors which, when activated, induce an increase in IL-8 secretion (Yu &Chadee, 1999). IL-8 is a chemokine that plays a dual role: At the onset of colonic inflammation, it attracts and activates neutrophils to cause nonspecific tissue damage. In contrast, at late progressive states of colitis EP4 signaling maintains normal mucosal integrity, promotes healing (Kabashimaet al., 2002) and suppresses colitis through upregulation of an anti-inflammatory cytokine, IL-10 (Nitta et al., 2002).

In smooth muscle cells, EP1 receptor-mediated intracellular Ca^{2+} mobilization leads to smooth muscle cells contraction, while EP2 and EP4 receptor-mediated increase in cytoplasmic cAMP levels cause relaxation (Narumiya et al., 1999).

PGE2 was shown to affect, in a dose dependent manner, the activity of the Na^{+}/K^{+} ATPase in cardiomyoctes (Skayian & Kreydiyyeh, 2006).

H. Protein Kinase A:

The cAMP-dependent protein kinase or simply Protein kinase A (PKA) is a serine/threonine kinase that regulates many cellular processes such as metabolism (Krebs et a., 1979), gene regulation (Roesler et al., 1988), cell growth and division (Boynton et al., 1983), cell differentiation (Liu, 1982), and ion channel's conductivity (Li et al., 1993).

The enzyme is a tetramer of two catalytic subunits (C) and two regulatory (R) subunits. The binding of two cAMP molecules generated from ATP by adenylate cyclase (AC) to each R subunit results in the release and activation of the catalytic subunits (Skalhegg &Tasken, 2000). G proteins are common regulators of AC and consequently of PKA. Sphingosine-1-phosphate (S1P) activates many types of G proteins, including Gi/o leading to AC inhibition and cAMP reduction. Other than PKA, cAMP activates ion channels directly like the class of cyclic nucleotide gated ion channels (Nakamura & Gold, 1987). It also activates the guanine nucleotide exchanging factors Epac1 and Epac2 (also named cAMP-GEFI and –II), which are specific activators of the small GTPase Rap1 (Schmidt et al., 2013). The cAMP pathway interacts with other intracellular signaling pathways, including cytokine and Ras-Raf-ERK pathways (Yu et al., 2013). Degradation of cAMP is mediated by cAMP

phosphodiesterases (PDEs) that hydrolyze cAMP into adenosine 5′-monophosphate (Omori &Kotera, 2007).

There are two distinct isoforms of PKA, PKA-I and PKAII that differ in their regulatory subunits, RI and RII respectively. PKA-I has high affinity for cAMP, is cytoplasmic and associated with growth and proliferation, whereas PKA-II enzyme has lower affinity for cAMP, is anchored to subcellular structures and compartments, and associated with increased differentiation and decreased proliferation.

The RI and RII amino terminal contains a dimerization site for interaction with the C subunit, and the carboxy terminal contains two cAMP binding sites. Each R subunit has two different isoforms, α and β (RIα, RIβ, RIIα, and RIIβ) (Skalhegg &Tasken, 2000). RIα has ubiquitous distribution, while RIβ is expressed primarily in brain, testis and B- and T-lymphocytes. Similarly, RIIα has ubiquitous distribution, while RIIβ is expressed in brain, adipose and some endocrine tissues.

The catalytic subunit (C) has two major isoforms Cα and Cβ (Skalhegg & Tasken, 2000). It has two lobes: the amino-terminal lobe (N-lobe) involved in MgATP binding, and carboxy terminal lobe (C-lobe) that contains the catalytic core and substrate docking sites. The C-subunit is also flanked by an N-terminal tail (N-tail) and a C-terminal tail (C-tail), which are anchored to the N and C-lobes of the core (Kim et al., 2007). The C-tail is an integral part of the active enzyme (Knighton et al., 1991).

PKA enzymes are confined to specific cellular microdomains by kinase anchoring proteins, AKAPs (Beene & Scott, 2007). AKAPs act as scaffold proteins that bind the regulatory subunits of the PKA, and lead it to specific locations within the cell where it can be close to its substrate (Gold et al., 2006).

One of the major nuclear targets of PKA is the transcription factor cAMP response-element-binding protein (CREB) (Mayr & Montminy, 2001). CREB targets up to 4000 genes involved in several cellular processes. CREB proteins bind optimally to palindromic CREs (sequence TGACGTCA) in promoters and upon phosphorylation by PKA they recruit the coactivator, CREB binding protein (CBP) to the promoter inducing cellular gene expression (Sands & Palmer, 2008).

Elevated cAMP levels in MDAMB-231 breast cancer cells and in response to leptin, exerted apoptotic effects. They abrogated both ERK1/2 and STAT3 phosphorylation, lowered protein levels of both regulatory RIα and catalytic subunits of PKA, reduced CREB phosphorylation, and inhibited both leptin-induced proliferation and migration (Naviglio et al., 2009). cAMP/PKA also regulate cell migration and actin dynamics, by targeting structural proteins, like actin, integrins, VASP and myosin light chain and regulatory proteins, like Rho GTPases, Src kinases, p21- activated kinases, phosphatases and proteases (Howe, 2004).

Cellular processes are regulated by a network of signaling pathways that interact and cross talk with each other, potentiating or antagonizing their effects. Thus the effect of cAMP/PKA depends also on what other pathways are activated or signaling molecules present. For example, isoproterenol (Edgar et al., 2002) promotes myocardial contraction by increasing the concentration of cAMP and decreasing cGMP levels. While the antidepressant medications fluoxetine and amitriptyline (Reierson et al., 2009), act by stimulating both cGMP and cAMP production. In human platelets, forskolin, which is an activator of AC, increases the production of cAMP which enhances NOS activity leading to NO synthesis (Russo et al., 2004).

Cyclic AMP can have opposite effect on NF-kB. While the transcription factor is activated in the anti-inflammatory response by PKA phosphorylation of IκB kinase, it (Zandi et al., 1997) is inhibited by cAMP in human monocytes and endothelial cells (Ollivier et al., 1996).

Cyclic AMP and Ca2+ signaling pathway are intertwined and may work together to enhance their individual effect or may act in an antagonistic manner. Increased intracellular calcium was reported to disrupt the vascular endothelial barrier, while high levels of cAMP enhance endothelial barrier functions. During inflammation increased cytosolic calcium induces a decrease in cAMP levels to promote the formation of intercellular gaps (Moore et al., 1998). In MIN6 β-cells, cAMP and Ca_{2+} signaling pathways cooperate to regulate insulin secretion (Landa et al., 2005).

PKA may alter also the activity of various ATPases and affect thus ion transport across the membrane. In COS-7 cells, phosphorylation of Na^+/K^+ ATPase was enhanced by treatment with PKA activator. PKA is also able to phosphorylate H+ /K+ -ATPase increasing its expression and cell surface localization (Poulsen et al., 2010).

CHAPTER III

MATERIALS AND METHODS

A. Materials:

FTY720P (FTY720P), Glyco-SNAP1, Carboxy-PTIO, anti-EDG-1, 3, 5, 6 and 8 rabbit polyclonal antibodies, goat anti-rabbit horse raddish peroxidase (HRP) conjugated IgG were purchased all from Santa Cruz Biotechnology, CA, USA.

PF-04418948 was purchased from Cayman Chemical Company, Michigan, USA.

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

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

Biorad assay and protein reagent, nitrocellulose membranes and western blotting luminol reagent were obtained from Biorad, California, USA.

Prostaglandin (PGE2), ouabain, indomethacin, Dulbecco's Minimal Essential Medium (DMEM) with 4500mg/L glucose and pyridoxine HCL, trypsin-EDTA, Penicillin/Streptomycin, Fetal Bovin Serum (FBS), 10x Phosphate Buffered Saline (PBS) without magnesium and calcium, and Adenosine 5′-triphosphate disodium salt (ATP), 2'-O-Dibutryryladenosine 3′,5′-cyclic monophosphate sodium salt (dbcAMP) were procured all from Sigma, Chemical Co, St Louis Missouri, USA.

The Human colonic ADENOCARCINOMA Cell line, Caco2, was purchased from American Type Culture Collection (ATCC).

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

B. Methods

1. Culture of Caco2 cells

Caco2 cells at passages 32-50 were grown in DMEM supplemented with 1% penicillin (100µg/ml) and streptomycin (100µg/ml) and 10% FBS in six well plates at a density of 1000 000 cells per well. The cells were kept in humidified incubator (95% O2, 5% CO2) at 37°C and treated at 85-90% confluence after an overnight starvation.

2. Treatment of Caco2 cells:

In every treatment, when the agent tested was dissolved in a solvent other than water, an equal volume of the vehicle was added to the control.

a. Effect of FTY720P on the Na^+/K^+ ATPase activity

Following starvation, Caco2 cells were treated for 15 minutes with 7.5 nM FTY720P. An equal volume of the vehicle DMSO was added to the control. The cells were then washed with PBS buffer (pH=7.4), scraped in lysis buffer to which protease inhibitors were added (9.9 ml of 150mM histidine buffer pH=7.4, 400 µl protease inhibitor (1 tablet/2ml H2O), 100 µl Triton-X (1mg/mlH_2O)), homogenized for 30 seconds using PRO Homogenizer at maximum speed (30,000 rpm), and centrifuged for 30 min at 20000 g and 4°C. Proteins in the supernatant were quantified calorimetrically at a wavelength of 595 nm using the Bradford method and then used to assay for the ATPase activity or for western blot analysis.

b. Determination of the S1P receptors mediating FTY720P effect

In order to determine the type of S1P receptors involved in the FTY720P effect, S1PR2 and S1PR3 were blocked respectively with JTE-013 (1 µM in DMSO) and CAY10444 (17.4 µM in DMF). The blockers were added to the cells 15 min before FTY720P.

The effect of specific agonist to the two receptors namely CYM5520 (2.5 µM in DMSO, S1PR2 agonist) and CYM5541 (2 µM in DMSO, S1PR3 agonist) was tested by adding them individually to the cells which were then incubated at 37°C for 15 min.

c. Involvement of PGE2 and determination of the EP receptors involved

The involvement of PGE2 was determined by treating the cells 1 hour prior to FTY720P with indomethacin, a COX inhibitor (100 µM, DMSO) or by direct treatment with exogenous PGE2 (1nM in alcohol, 15 min).

To determine the type of EP receptor mediating PGE2 effect, EP2 receptors were blocked with PF-04418948 (1 µM in DMSO, 30 min before PGE2). EP3 receptors were activated by the agonist sulprostone (1 µm for 15 min).

d. Involvement of NO

Involvement of NO was tested by using the nitric oxide scavenger carboxy-PTIO (30µM in water) or by using the nitric oxide donor Glyco-SNAP-1 (2 µM) .The cells were incubated with carboxy-PTIO for one hour before addition of FTY720P.The incubation with SNAP was for 15 min.

e. Involvement of PKA

Since receptors for S1P and PGE2 may be coupled to Gi or Gs, cAMP may be a potential mediator in the effect of S1P on the pump. The involvement of cAMP/PKA was tested by treating the cells with an inhibitor of PKA namely RpcAMP added1 hour

prior to FTY720P (30 µM in water) or by treating the cells with dbcAMP (10 µM in water), a cell permeable cAMP analogue for 15 min.

f. Testing the involvement of PKC and PI3K

Both S1P and PGE2 receptors may be coupled to Gq proteins that activate PLC and eventually PKC. The involvement of PKC in the signaling pathway was tested by addition of a PKC inhibitor, calphostin C (50 nM in DMSO) 1 hour before FTY720P or by treating the cells with PKC activator, PMA (100nM in DMSO) for 15 min. PI3K involvement was tested using its inhibitor wortmannin (100 nM in DMSO) added 1hour before FTY720P.

g. Locating the involved mediators with respect to each other in the signaling pathway

To know if PKC is upstream or downstream of PGE2, Caco2 cells were treated with PMA, a PKC activator in presence of indomethacin, an inhibitor of PGE2 synthesis and with calphostin, a PKC inhibitor, in presence of PGE2.

To determine the position of PI3K and NO relative to PGE2, cells were treated with PGE2 in presence of an inhibitor of PI3K (wortmannin) or a nitric oxide scavenger (PTIO). The inhibitors were added to the cells1 hour prior to PGE2. The cells were then incubated with PGE2 in presence of the inhibitors for an additional 15 min period.

3. The Na^+/K^+ ATPase Activity Assay

After each of the above mentioned treatments, cells underwent scraping after addition of lysis buffer, homogenization in the presence of histidine and quantification of the protein content. Cell homogenates were then diluted to a concentration 0.5 µg/µl with histidine buffer (pH 7.4, 150mM). A homogenate volume of 65 µl was incubated with 17 µl of 1% saponin for 15 min at room temperature, followed by a 15 min

incubation with a phosphatase inhibitor cocktail (300µl of pyrophosphate (200mM), 300µlglycerophosphate (200mM), and 400µl of water). Then 12 µl were taken from each sample and incubated with a mixture of NaCl (1240mM, 10µl), KCl (200mM, 10µl), $MgCl_2$ (40 mM, 10 µl), ATP (30mM, 10 µl), and 20 µl of histidine buffer for 30 min at 37°C with or without ouabain (15 mM, 30 µl). When ouabain was omitted, it was replaced with 30 µl water. The reaction was stopped by addition of 10µl 50% Trichloroacetic acid.

The samples were then spun at a speed of 16.163 x g for 5 min and 90 µl were taken from the supernatant and added to 80 µl of Ferrous sulfate-molybdate reagent (made of 0.5 mg Ferrous sulfate, 1 ml of ammonium molybdate (100 g/L in 10N H_2SO_4), and 9 ml of water). This mixture gave a blue color reflecting the concentration of inorganic phosphate liberated which was quantified in a microplate reader at a wavelength of 750nm.

4. Western Blot Analysis

Fourty micrograms of proteins were run on 10% SDS polyacrylamide gels (To 133 ml of 30% polyacrylamide were added 100 ml of Tris-HCl (1.5 M, pH= 8.8), 4 ml 10% SDS and 163 ml water) and then transferred to nitrocellulose membranes. The membranes were then blocked for 40 min with blocking buffer (1L of 1x PBS, 1ml of Tween20, 30 g skimmed milk, and 10 ml 0.1% sodium azide), and then cut into strips incubated each overnight at 4°C with primary anitbodies to the different S1P receptors: anti-EDG1rabbit polyclonal antibodies, anti-EDG5 rabbit polyclonal antibodies, anti-EDG3rabbit polyclonal antibodies, anti-EDG6 rabbit polyclonal antibodies, anti-EDG8 rabbit polyclonal antibodies. The membranes were incubated with goat anti-rabbit HRP

conjugated IgG secondary antibodies for 1 hour at room temperature. The signal was detected by chemiluminescence by adding 1 ml luminol Clarity ECL Substrate .The intensity of the signal was detected using ChemiDoc^{TM}MP.

5. Statistical Analysis

The data were tested for statistical significance using a one-way analysis of variance followed by a Tukey-Kramer multiple comparison test using GraphPad InStat 3. The results are reported as mean ± SEM.

CHAPTER IV

RESULTS

A. Expression of S1P receptors in Caco2 cells:

The expression and abundance of the different types of S1P receptors was determined by western blot analysis. The results revealed that S1PR2 is the most abundantly expressed expressed receptor followed by S1PR3 (Fig 1). S1PR1 is mildly expressed, while S1PR4 and S1PR5 were not detected under experimental conditions.

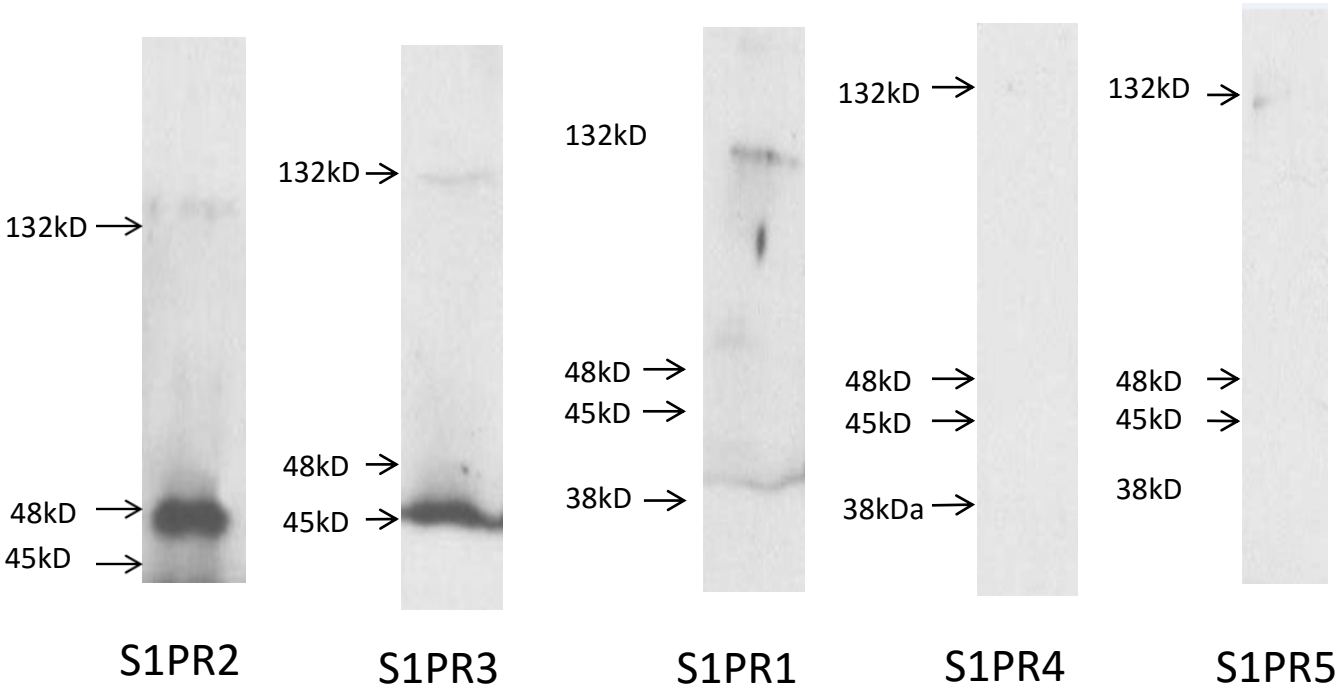


Fig 2. S1P receptors' expression in Caco-2 cells. Protein loaded: 40µg

B. Time response study on the effect of FTY720P on the Na+/K+ATPase in Caco-2 cells

Caco-2 cells treated with FTY720P (7.5nM) for different time periods exhibited a lower Na^+/K^+ ATPase activity. The highest inhibitory effect was observed at 15min,

with a decrease of around 50% as, as compared to its corresponding control value (Fig 2).

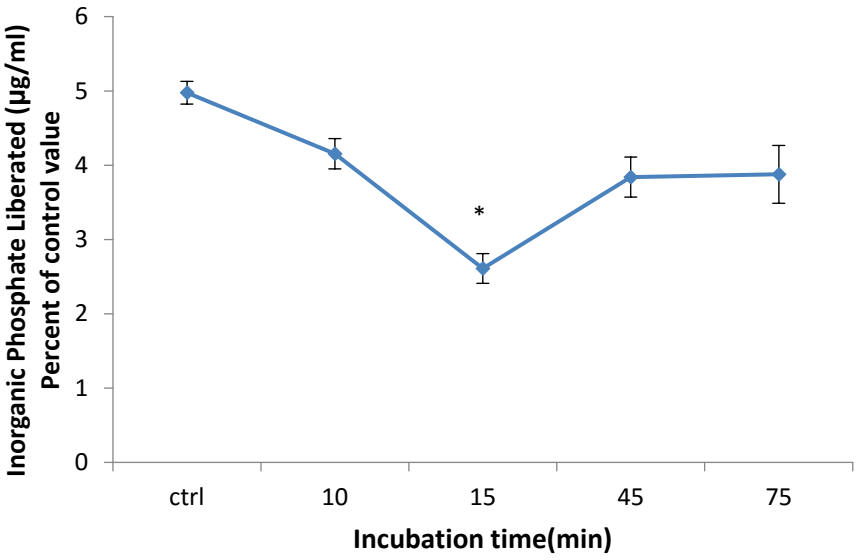


Fig 3. The highest inhibitory effect of FTY720P (7.5nM) on Na^+/K^+ ATPase appeared at 15min. Values are means ± SEM of 3 observations.* significantly different from the control at $p<0.01$, as indicated by ANOVA followed by tukey test.

C. Determination of the type of S1PR involved in the effect of FTY720P on the Na^+/K^+ ATPase

The inhibitory effect of FTY720P on the Na^+/K^+ATPase was not observed when S1PR2s were blocked with JTE-013, but still appeared when cells were treated simultaneously with Cay-10444 a specific antagonist of S1PR3 (Fig 3).

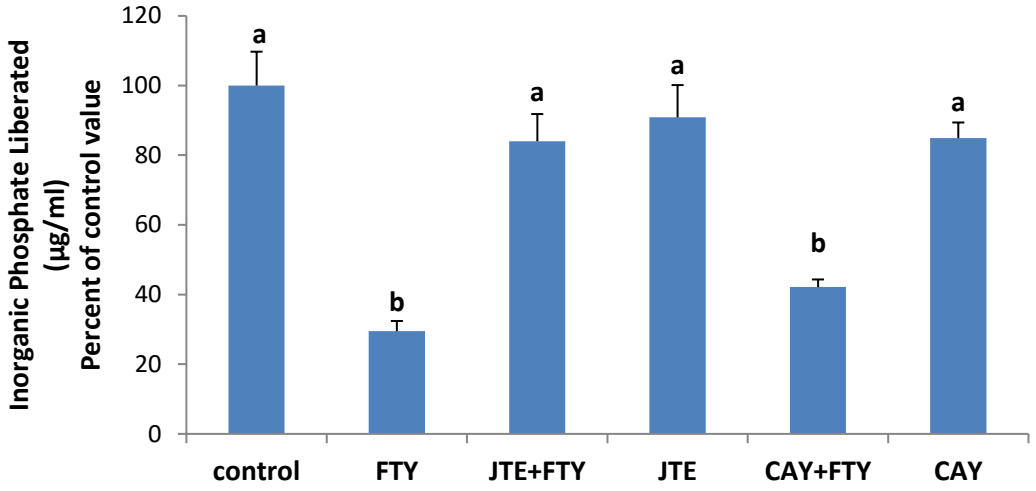


Fig 4. Effect of FTY720P on the pump in presence of JTE-013(1 µM in DMSO), a blocker of S1PR2, or CAY10444 (17.4 µM in DMF), a blocker of S1PR3. An equal volume of the vehicle(s) was added to the control. Values are means ± SEM of 3 observations. Bars not sharing the same letter are considered significantly different from each other at $p<0.05$, as indicated by ANOVA followed by tukey test. FTY=FTY720P; JTE=JTE-013; CAY= CAY 10444.

CYM5520, an agonist of S1PR2, mimicked the effect of FTY720P on the Na^+/K^+ ATPase while CYM5541, an antagonist of S1PR3, did not exert any effect on the pump (Fig 4).

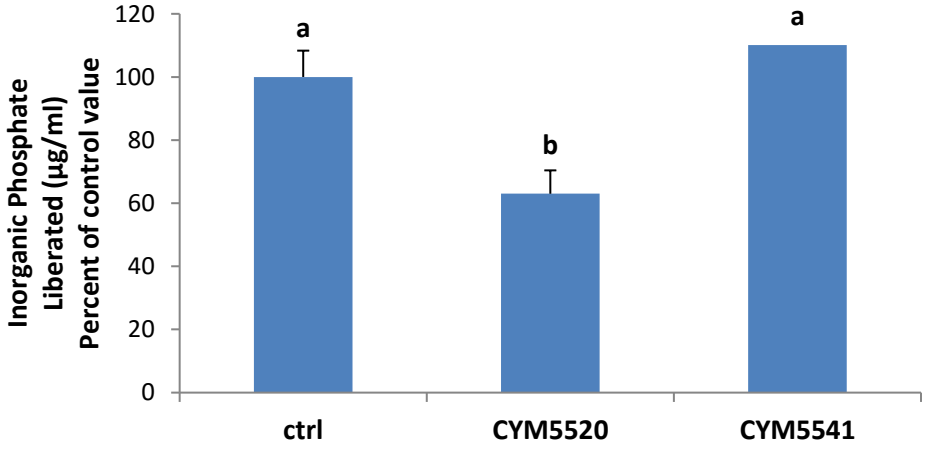


Fig 5. Effect of CYM5520, a S1PR2 agonist (2.5 µM, 15 min) and CYM5541, a S1PR3 agonist (2 µM, 15 min) on the activity of the Na^+/K^+ ATPase. An equal volume of the vehicle DMSO was added to the control.Values are means ± SEM of 3 observations. Bars not sharing the same letter are considered significantly different from each other at $p<0.05$, as indicated by ANOVA followed by tukey test.

D. Involvement of PGE2 in the effect of FTY720P on the Na^+/K^+ ATPaseand determination of the type of EP receptor activated

In a previous work, PGE2 was shown to affect pump's activity in HepG2 cells via PGE2. To test the involvement of the prostaglandin in Caco-2 cells, PGE2 production was inhibited with indomethacin, an inhibitor of COX enzymes. In presence of indomethacin, FTY720P could not exert any effect on the pump. Treating cells with exogenous PGE2, reduced significantly, similarly to FTY720P, the activity of the Na^+/K^+ ATPase (Fig 5).

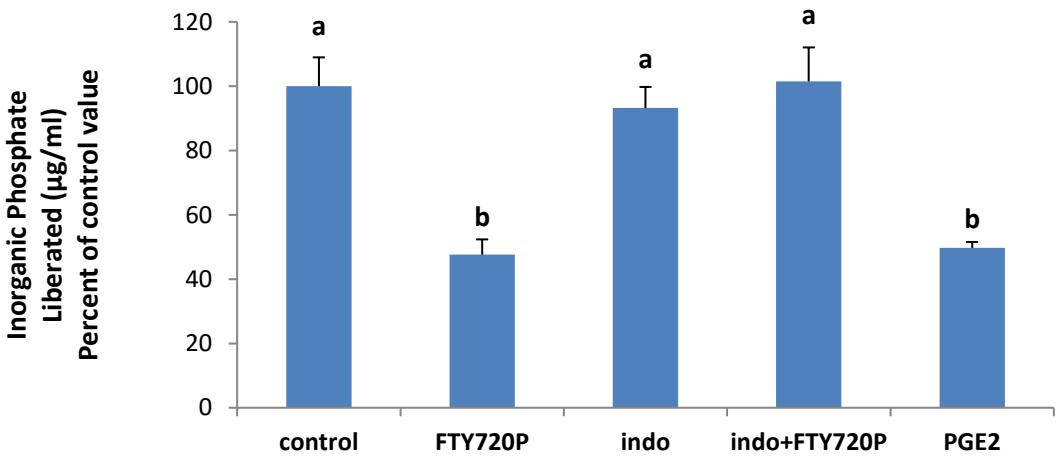


Fig 6. PGE2 is a mediator of the inhibitory effect of FTY720P on the Na^+/K^+ ATPase. In presence of indomethacin the effect of FTY720P did not appear, while PGE2 produced a similar inhibitory effect to FTY720P.Values are means ± SEM of 3 observations. Bars not sharing the same letter are considered significantly different from each other at $p<0.05$, as indicated by ANOVA followed by tukey test. indo= Indomethacin. Anova followed by tukey test.

PGE2 is known to act via 4 types of EP receptors that activate different signaling pathways. The FTY720P-induced inhibition of the pump was still observed when EP2 receptors were blocked with PF-04418948. Treating the cells with the EP3 receptor agonist sulprostone reduced significantly the activity of the ATPase (Fig 6).

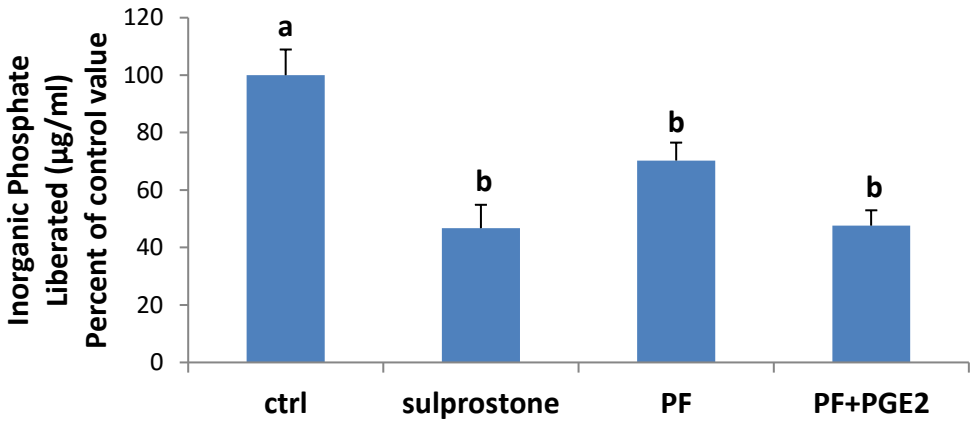


Fig 7. PGE2 activates EP3 receptors. Values are means ± SEM of 3 observations. Bars not sharing the same letter are considered significantly different from each other at $p<0.05$, as indicated by ANOVA followed by tukey test. PF= PF-04418948.

Depending on the receptors activated, PGE2 may increase or decrease cAMP levels and thus may activate or inhibit PKA. To check for any role of PKA in the PGE2 effect, Caco-2 cells were treated before PGE2 with RpcAMP, a PKA inhibitor, or with dibutyryl cAMP, a cell permeable analogue of cAMP. dbcAMP had no effect on the Na^+/K^+ ATPase while RpcAMP alone exerted an inhibitory effect. PGE2 still exerted its effect when PKA was inhibited with RpcAMP (Fig 7).

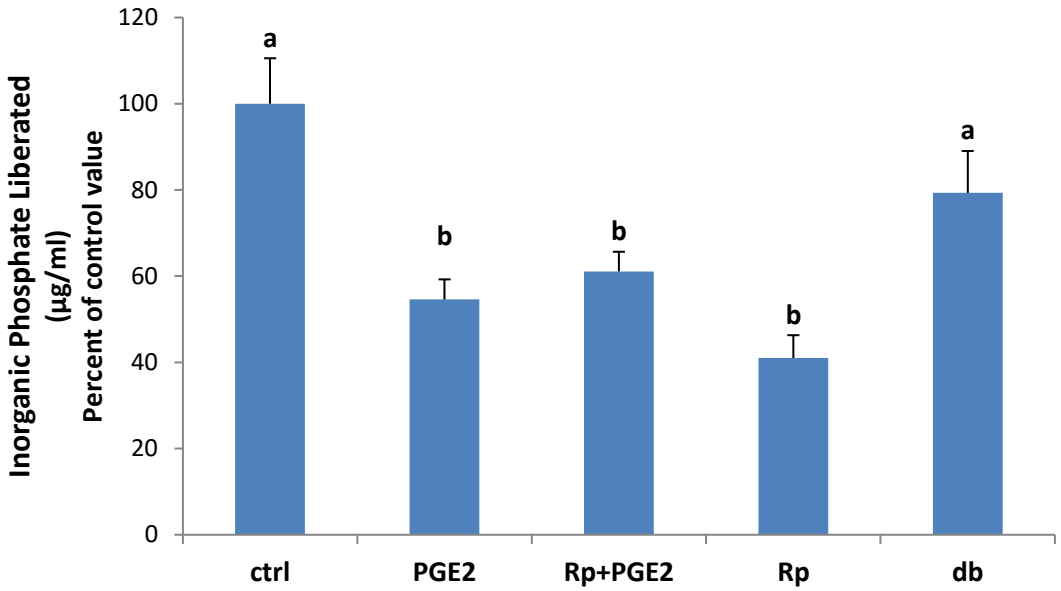


Fig 8. Involvement of PKA in the effect of PGE2 on the Na^+/K^+ ATPase. Values are means ± SEM of 3 observations. Bars not sharing the same letter are considered significantly different from each other at $p<0.05$, as indicated by ANOVA followed by tukey test. Rp=RpcAMP; db= dibutyrylcAMP.

E. PKC involvement in FTY720P effect on the Na^+/K^+ ATPase activity

Since FTY720P was shown to act via S1PR2 receptor, and since the latter couples to Gq and Gi/o which may both activate PLC, the involvement of PKC in the effect of FTY720P was investigated. Calphostin, a PKC inhibitor, abolished the inhibitory effect of FTY720P on the Na^+/K^+ ATPase, while PMA, a PKC activator reduced significantly the activity of the ATPase as FTY720P did (Fig 8).

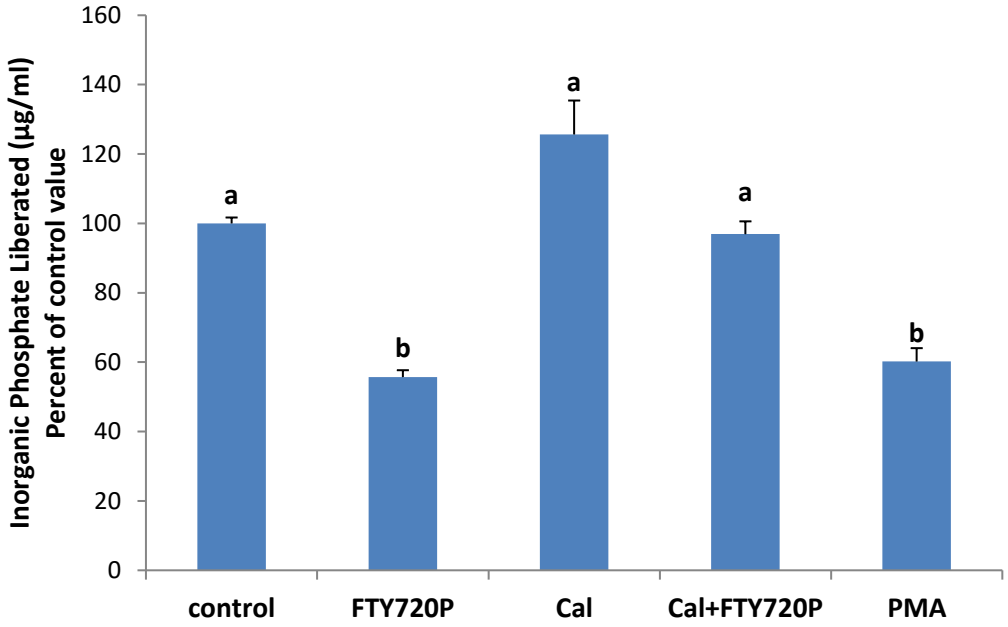


Fig 9. FTY720P exerts its effect on the pump via a stimulation of PKC. Calphostin (50 nM, 1 hr) abolished the effect of FTY720P while PMA (100nM, 15min) mimicked it. An equal volume of the vehicle DMSO was added to the control.Values are means ± SEM of 3 observations. Bars not sharing the same letter are considered significantly different from each other at $p<0.05$, as indicated by ANOVA followed by tukey test. Cal= Calphostin

F. PI3K mediates the effect of FTY720P on the Na^+/K^+ ATPase activity

The effect of FTY720P did not appear in presence of wortmannin, a PI3kinase inhibitor (Fig 9).

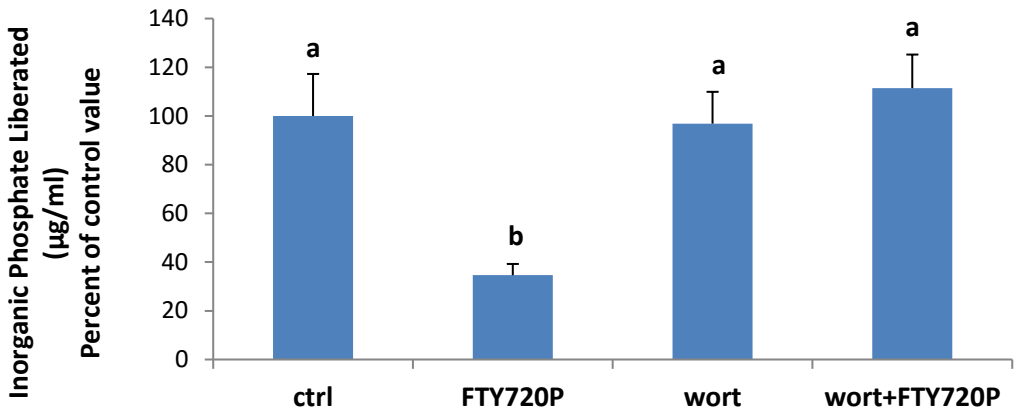


Fig 10.PI3K is involved in the signaling pathway of FTY720P on the pump. The effect of FTY720P did not appear in presence of wortmannin (100 nM, 1hr), an inhibitor of PI3K. An equal volume of the vehicle DMSO was added to the control.Values are means ± SEM of 3 observations. Bars not sharing the same letter are considered significantly different from each other at $p<0.05$, as indicated by ANOVA followed by tukey test. wort= wortmannin.

G. Involvement of nitric oxide in the effect of FTY720P on the Na^+/K^+ ATPase

Preventing NO accumulation with the NO scavenger, carboxy-PTIO, eliminated FTY720P's effect on the pump. However, treating the cells with the nitric oxide donor, Glyco-SNAP-1, mimicked the effect of FTY720P on the Na^+/K^+ ATPase (Fig 10).

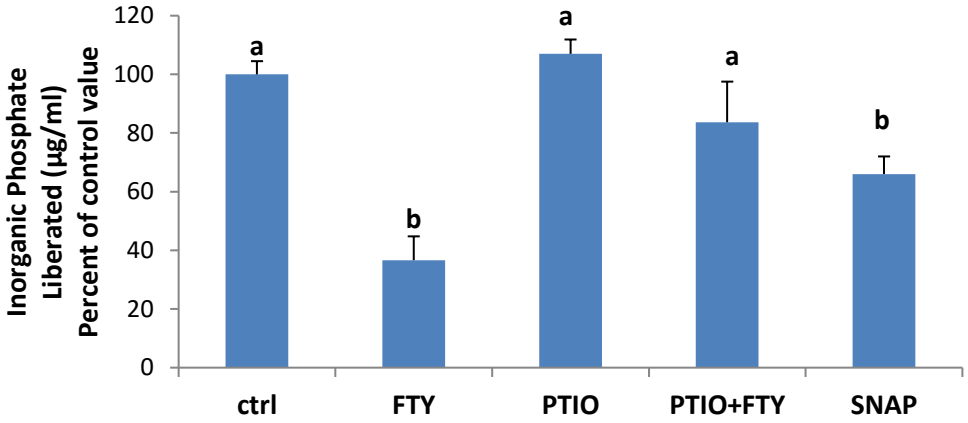


Fig 11.Nitric oxide mediates the inhibitory effect of FTY720P on the pump. Values are means ± SEM of 3 observations. Bars not sharing the same letter are considered significantly different from each other at $p<0.05$, as indicated by ANOVA followed by tukey test. FTY= FTY720P; PTIO= Carboxy-PTIO; SNAP= Glyco-SNAP-1.

H. PKC location relative toPGE2:

To locate PKC relative to PGE2, cells were treated with PGE2 in the presence of a PKC inhibitor, calphostin. PGE2 effect was unchanged in presence of the inhibitor (Fig 11).

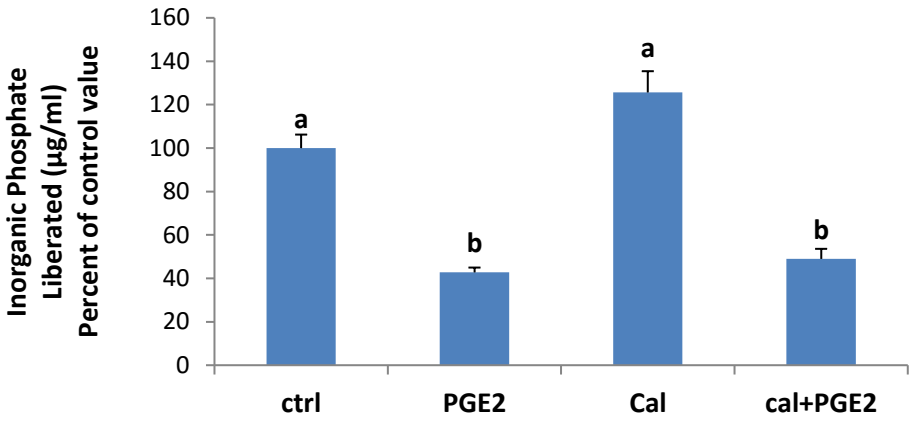


Fig 12. PKC is upstream of PGE2. The effect of PGE2 on the ATPase was not affected by calphostin (50 nM, 1 hr), an inhibitor of PKC. An equal volume of the vehicle DMSO was added to the control.Values are means ± SEM of 3 observations. Bars not sharing the same letter are considered significantly different from each other at $p<0.01$, as indicated by ANOVA followed by tukey test. Cal= Calphostin.

PMA, a PKC activator, reduced the activity of the Na^+/K^+ ATPase. This inhibitory effect was not manifested in presence of indomethacin, an inhibitor of the the COX enzymes responsible for PGE2 production (Fig 12).

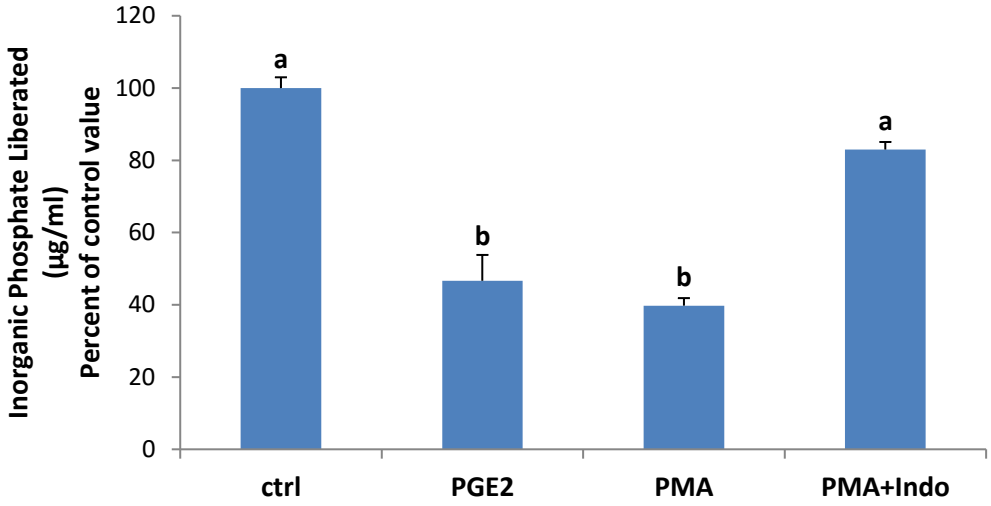


Fig 13.PGE2 is downstream PKC. Values are means ± SEM of 3 observations. Bars not sharing the same letter are considered significantly different from each other at $p<0.05$, as indicated by ANOVA followed bu tukey test. Indo= indomethacin

I. PI3K location relative to PGE2

To locate PI3K relative to PGE2, cells were treated with the prostaglandin in presence of wortmannin, an inhibitor of PI3K.In presence of the inhibitor, the effect of PGE2 still appeared (Fig 13).

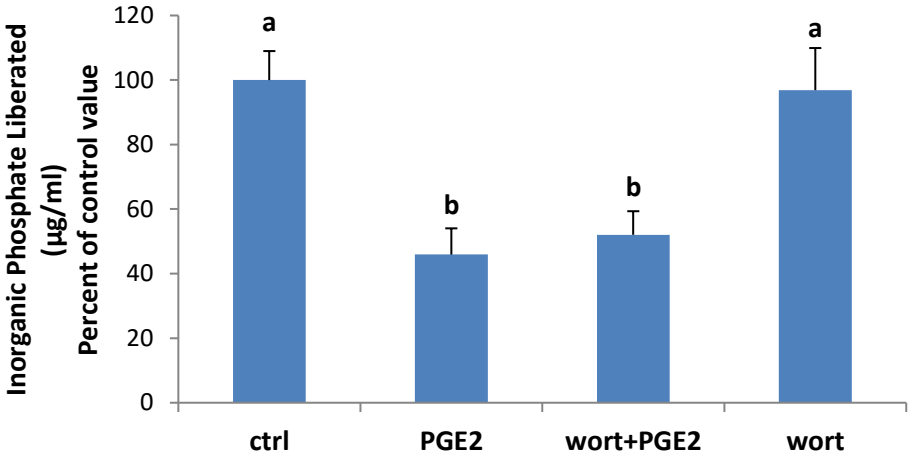


Fig 14.PI3K is upstream PGE2. Values are means ± SEM of 3 observations. Bars not sharing the same letter are considered significantly different from each other at $p<0.05$, as indicated by ANOVA followed by tukey test.wort= wortmannin.

J. NO location relative to PGE2

The effect of PGE2 disappeared when cells were treated simultaneously with the NO scavenger PTIO (Fig 14).

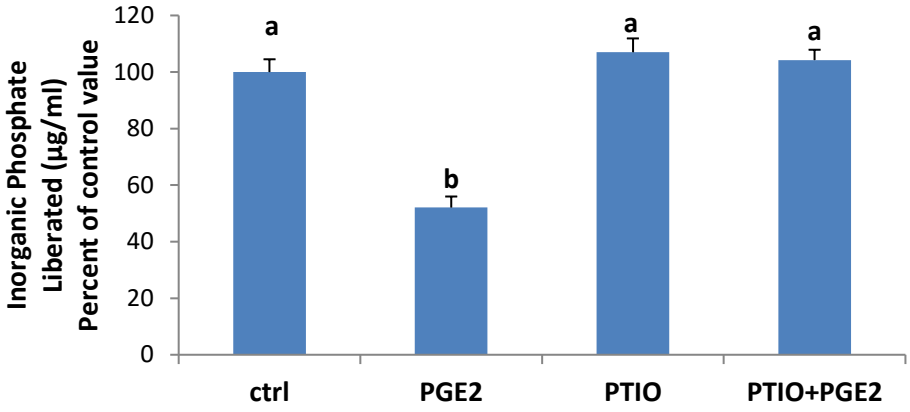


Fig 15.NO is downstream PGE2. In presence of PTIO (30µM, 1 hr), the effect of PGE2 was not manifested. Values are means ± SEM of 3 observations. Bars not sharing the same letter are considered significantly different from each other at $p<0.01$, as indicated by ANOVA followed by tukey test. PTIO= Carboxy-PTIO.

K. PKC position with respect to PI3K

The inhibitory effect of PMA, an activator of PKC, did not appear in presence of wortmannin, an inhibitor of PI3K. Wortmannin alone did not have any effect on the pump (Fig 15).

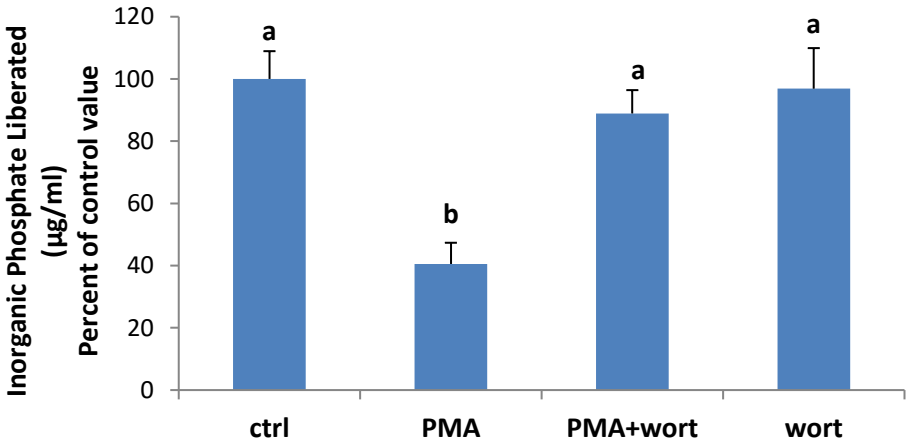


Fig16.PKC is upstream PI3K. Values are means ± SEM of 3 observations. Bars not sharing the same letter are considered significantly different from each other at $p<0.05$, as indicated by ANOVA followed by tukey test. wort=wortmannin.

CHAPTER V

DISCUSSION

The Na^+/K^+ ATPase plays a pivotal role in the absorption of sodium and consequently water from the colon. Alteration in its activity may cause diarrhea or constipation. The diarrhea that appears in inflammatory bowel disease is accompanied by a lower activity of the Na^+/K^+ ATPase and higer levels of S1P. Thus this work was undertaken to investigate any possible relationship between the sphingolipid and the ATPase, using Caco-2 cells as a model and FTYY720P as an analogue of S1P. Caco2 cells express the same transporters as normal colon cells, so they can be used as a model for sodium transport (Calcagno et al., 2006).

A. FTY720P exerts a maximal inhibitory effect on the Na^+/K^+ ATPase at 15min.

Previous work revealed in HepG2 liver cells, a significant inhibitory effect of S1P (Dakroub & Kreydiyyeh, 2012) and FTY720P (Al Alam & Kreydiyyeh, 2016) on the Na^+/K^+ ATPase that was maximal at 15min.This work showed the existence of a similar trend in colon cells. In Caco-2 cells again FTY720P reduced the ATPase activity to almost half its control value at 15 min, indicating that such an effect is not tissue specific.

B. FTY720P inhibition of the Na^+/K^+ ATPase is mediated via S1PR2

S1P and its analogue FTY720P are known to exert their effect via five different S1P receptors (S1PR1-5). Although it is stated in the literature that FTY720P can bind 4 of the 5 S1P receptors (S1PR1, S1PR3, S1PR4, and S1PR5) (Murphy et al., 2012),

previous work in our lab showed that FTY720P can bind to S1PR2 in HepG2 cells (Al Alam & Kreydiyyeh, 2016). Antibodies against each type of S1PR were used to detect their expression in Caco2 cells. Western blot analysis (Fig1) showed that S1PR2 has the highest expression followed by S1PR3. S1PR4 and S1PR5 are not expressed while S1PR1 is slightly expressed.

Similar results were reported in two recent studies whereby S1PR2 was found to be the most abundant receptor present in normal intestinal epithelial cells; all other receptors except S1PR4 were also expressed but at lower levels (Chen et al., 2017). In these cells, S1PR2 was found to suppress tumors by blocking the cell cycle and the de-differentiation process (Petti et al., 2017).

To determine which S1PR is mediating the effect of FTY720P on the pump, agonists and antagonists for S1PR2 and S1PR3 were used, since these were the only receptors that appeared to be expressed in Caco2 cells. Inhibiting S1PR2 (using JTE-013) restored the activity of the ATPase to control values, while inhibiting S1PR3 (using Cay-10444) maintained the pump's inhibition by FTY720P (Fig 3). In addition, using CYM5520, an agonist of S1PR2, mimicked FTY720P's effect on the Na^+/K^+ ATPase while CYM5541, an agonist of S1PR3, did not exert any effect on the ATPase (Fig 4). The results suggest that FTY720P acts on the ATPase via activation of S1PR2. A similar involvement of S1PR2 was reported in the inhibitory effect of FTY720P on the ATPase in HepG2 cells (Al Alam & Kreydiyyeh, 2016).

C. ProstaglandinE2 (PGE2) mediates the effect of FTY720P on the Na^+/K^+ATPase and acts via EP3

Several studies showed that S1P induces, in different cell types, PGE2 production by enhancing COX-2 expression. This is why investigating the involvement of PGE2 in FTY720P's effect on the Na^+/K^+ ATPase activity was deemed necessary. Caco-2 cells treated with exogenous PGE2 had a lower Na^+/K^+ATPase activity, while inhibiting PGE2 production with indomethacin abolished the effect of FT720P on the ATPase (Fig 5). It was concluded that PGE2 is a mediator in the signaling pathway through which FTY720P acts on the pump in colonic cells. In line with these findings, it was reported that PGE2 at picomolar concentrations exerted a 40% decrease in the Na^+/K^+ATPase activity (Cohen-Luria et al 1993), in a clone of Madin-Darby canine kidney (MDCK) cells.

PGE2 acts via 4 different G-protein coupled EP receptors that are all expressed in colonocytes (Shojiet al., 2004). Inhibiting here EP2 receptor with its antagonist PF-04418948, did not alter the inhibitory effect of PGE2 on the pump, while activating EP3 receptor with its agonist sulprostone, resulted in a similar inhibitory effect on the ATPase to that observed with PGE2 (Fig 6). The results indicate that PGE2 acts on the Na^+/K^+ pump by activating EP3 receptors.

EP3 receptors are coupled to Gi. They inhibit adenylate cyclase and reduce thus the level of cAMP and the activity of PKA (Shojiet al., 2004).To check for the involvement of PKA downstream of PGE2, Caco-2 cells were treated with dibutyrylcAMP, an analogue of cAMP or with PGE2 in presence of an inhibitor of PKA namely RpcAMP. PGE2 maintained its inhibition on the Na^+/K^+ ATPase when PKA was inhibited, while dbcAMP had no effect (Fig 7). RpcAMP alone, reduced similarly to PGE2, the activity of the pump, confirming the involvement of EP3, and the need to reduce PKA activity to observe the inhibitory effect of PGE2 on the pump.

D. PKC is a mediator in the signaling pathway, and is located upstream of PGE2

Our results showed that FTY720P acts via S1PR2s, which are coupled to Gi, G12/13 and Gq. Gq activates phospholipase C leading to an increase in the level of diacylglycerol and calcium and consequently an activation of PKC, a serine/threonine kinase that phosphorylates downstream targets (Adada et al., 2013). PKC was reported to modulate the Na^+/K^+ATPase activity either directly by phosphorylation (Feschenko et al., 2000) or indirectly by phosphorylating and activating other intermediate molecules that would eventually alter the ATPase activity. To test the involvement of PKC, cells were treated with PMA, an activator of PKC or with FTY720P in presence of calphostin, a PKC inhibitor. The inhibitory effect of FTY720P was mimicked by PMA but completely abolished by calphostin (Fig 8) implying that FTY720P activates PKC. The literature reports a similar dose-dependent effect of phorbol esters (PMA) on renal cortical Na^+/K^+ ATPase activity (Bełtowski et al, 2004).

Treating the cells with PGE2 in presence of calphostin did not abrogate the effect of the prostaglandin and still a reduction in the ATPase activity was observed, which indicates that PGE2 effect is not mediated through PKC (Fig 11). In addition, the inhibitory effect of PMA did not appear in presence of the COX inhibitor, indomethacin (Fig 12), indicating that PKC is upstream of PGE2. Such a role of PKC in PGE2 production has been observed by other scientists. In intestinal subepithelial myofibroblasts (SEMFs), PKC was shown to be involved in the S1P-induced COX-2 expression and PGE2 production (Ohama et al., 2008).

E. PI3K is a mediator in the FTY720P signaling pathway, and is located upstream of PGE2

Several studies showed that S1PR2 which couples to Gi, Gq and G12,signals via PI3K/Akt (Beckham et al., 2013). To determine if PI3K plays any role in the signaling pathway through which FTY720P acts on the Na+/K+ATPase, Caco-2 cells were treated with FTY720P in presence of an inhibitor of PI3K, namely wortmannin. In this case, no change was observed in the activity of the pump, suggesting an involvement of PI3K (Fig 9). The literature reports such an activation of PI3K/Akt by Gq and Gi. Murga et al (1998) demonstrated, in COS-7 cells an involvement of PI3K/Akt in the acetylcholine prevention of UV-induced apoptosis, a process that is mediated via m1 and m2 muscarinic receptors coupled respectively to Gq and Gi proteins. Yu et al. (2016) showed also that knockdown of S1PR2 in murine bone marrow-derived monocytes and macrophages treated with the oral pathogen Aggregatibacter actinomycetemcomitans, resulted in a lower activity of PI3K, ERK,JNK,p38 and NF-KB as compared to the control (Yu, 2016).

Not only S1PR2 but many other G12/13-, Gi/o-, and Gq-coupled receptors were shown to activate the (PI3K)/Akt pathway. Merighi et al. (2005) reported that activation of the A3 adenosine receptors which are coupled to Gi/Gq, inhibited cell proliferation by stimulating PI3K-dependent Akt phosphorylation (Merighi et al., 2005).

G protein coupled receptors can activate PI3K via their Gα or their Gβγ subunits (Schwindinger and Robishaw, 2001).

Gαi-dependent PI3K activation (Tanski et al., 2002) was found to be behind the S1P-induced smooth muscle cell migration. PI3K in turn can have a direct or an indirect effect on the Na^+/K^+ pump. Dopamine, which acts via GPCRs, was shown to

induce endocytosis of the Na^+/K^+ ATPase through PI3K-$I_A$which binds to a proline rich sequence in the alpha subunit of the ATPase that becomes accessible only upon phosphorylation of a serine residue upstream of the sequence by PKC (Yudowski et al., 1999). Inhibition of myocardial Na^+/K^+ ATPase during endotoxemia does not occur via a direct phosphorylation by PI3K but via activation of PI3K/Rac1/NADPH oxidase (Zhang et al., 2012).

To determine whether PI3K is upstream or downstream of PGE2, the cells were treated with the prostaglandin in presence of wortmannin, an inhibitor of PI3K. The inhibitory effect of PGE2 appeared unchanged in presence of wortmannin implying that PI3K is upstream of PGE2 (Fig 13). These findings are in line with those of Hsieh et al (2006) who showed in vascular smooth muscle cells, a S1P-induced increase in COX-2 expression that was mediated via PI3K/Akt pathway.

F. Nitric oxide (NO) is involved in the effect of FTY720P on the Na^+/K^+ ATPase and is located downstream of PGE2

The involvement of NO in the effect of PGE2 on the ATPase was tested by incubating the cells with Glyco-SNAP-1, a nitric oxide donor or with FTY720P in presence of carboxy-PTIO, a nitric oxide scavenger. The effect of FTY720P was mimicked by SNAP-1 and abrogated by PTIO, demonstrating thus a role of NO in the signaling pathway (Fig 10). In rat vascular smooth cells, however, S1P had an opposite effect and reduced the IL-1 induced NO generation (Machida et al, 2007).

A similar inhibitory effect of NO on the Na+/K+ATPase was observed in opossum kidney (OK) proximal tubule cell line (Liang M. & Knox F.G., 1999), in

mouse brain endothelial cells via cGMP/PKG dependent mechanisms (Pontiggia et al., 1998) and in HepG2 cells (Al Alam & Kreydiyyeh, 2016).

The effect of PGE2 on the Na^+/K^+ ATPase disappeared in presence of PTIO, indicating that NO is downstream of PGE2 (Fig 14). PGE2 exerts its effect on the pump through Gi-linked EP3 receptors that inhibit adenylyl cyclase (Hatae et al., 2002). An increase in cAMP levels was found to correlate with a reduced iNOS expression in LPS -stimulated primary astrocytes (Pahan et al., 1997) and C6 glioma cells (Won et al., 2001). Consequently, a decrease in cAMP levels via EP3 receptors is expected to increase NOS activity and NO levels.

G. FTY720P inhibits the ATPase through PI3K which is dependent on PKC activation

PMA, an activator of PKC, did not inhibit the pump in presence of wortmannin, an inhibitor of PI3K (Fig 15). So PKC is located upstream of PI3K in this pathway. These two molecules can lead to pump inhibition either directly or through other signaling molecules. protein kinase C phosphorylation of a serine residue in the Na^+/K^+ ATPase α subunit exposes a proline rich sequence to which PI3K-IA binds and induces Na^+/K^+ ATPase endocytosis (Yudowski et al., 2000). But in this study these two molecules affect the pump through PGE2, with PKC being upstream of PI3K. PKC could activate PI3K by increasing the availability of phosphatidylinositol-4, 5-bisphosphate (PIP_2), which recruits PI3K and acts as its substrate. PIP_2 availability depends on the myristoylated alanine-rich C kinase substrate (MARCKS) protein which sequesters PIP_2, but PKC phosphorylation of MARCKS at PIP_2-binding site reduces its

action, allowing PI3K activation (Ziemba et al., 2016). This hypothesis would need however to be verified.

Conclusion

In summary, FTY720P exerts an inhibitory effect on Na^+/K^+ ATPase in CaCo2 cells, as was seen before in HepG2 cells. FTY720P acts on the ATPase through S1PR2 and activates the signaling pathway summarized in Fig 16 below.

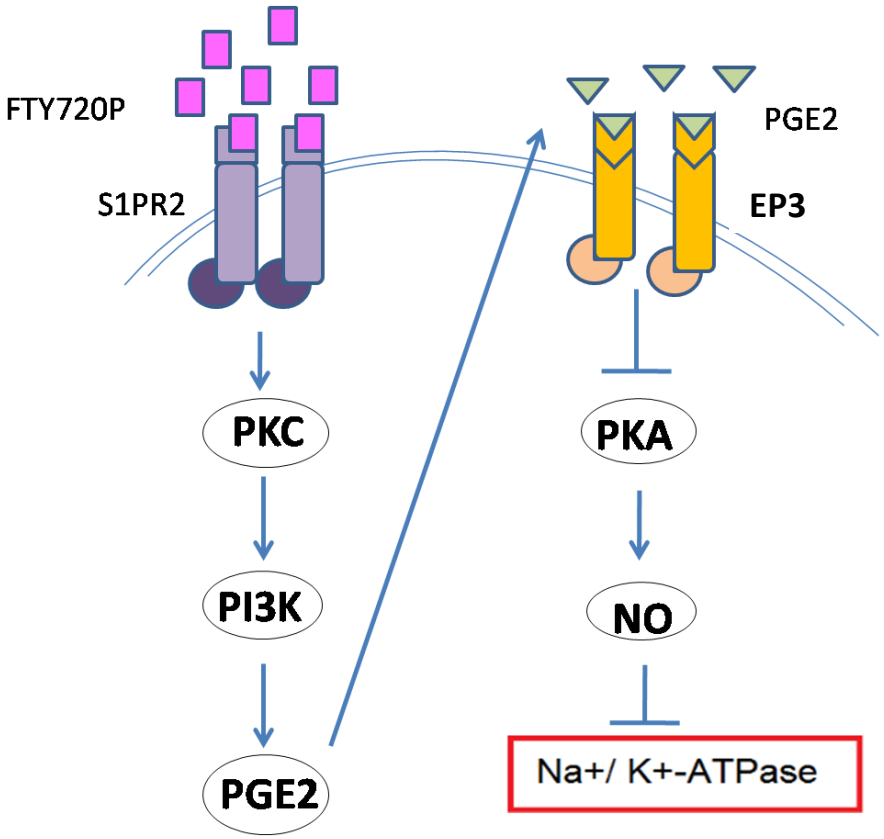


Fig 17. FTY720P inhibits the ATPase through this signaling pathway.

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