

AMERICAN UNIVERSITY OF BEIRUT

MEDIATORS OF THE INHIBITORY EFFECT OF
FTY720 PHOSPHATE, AN ANALOGUE OF SPHINGOSINE ONE
PHOSPHATE, ON HEPATIC Na⁺/K⁺ ATPASE

by

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AN ABSTRACT OF THE THESIS OF

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Title: Mediators of the inhibitory effect of FTY720 phosphate, an analogue of Sphingosine one phosphate, on hepatic Na⁺/K⁺ ATPase

An inhibitory effect of sphingosine -1- phosphate (S1P) on hepatic Na⁺/K⁺ ATPase was previously demonstrated. This work is an attempt to delineate the signaling pathway involved using its agonist FTY720P (FTY720P). HepG2 cells were used as a model and 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 inhibitor of the ATPase. FTY720P (7.5 nM, 15min) significantly inhibited the Na⁺/K⁺ATPase. This effect disappeared completely in presence of JTE-013, a specific blocker of S1P2 receptors. The inhibition was similarly abrogated by calphostin, indomethacin, RpcAMP, PTIO, an NF-κB inhibitor, and PD98059, respective inhibitors of protein kinase C (PKC), cyclooxygenases, protein kinase A (PKA), nitric oxide synthase (NOS), NF-κB, and extracellular signal-regulated kinase (ERK1/2). Treatment with prostaglandin E₂ (PGE₂) or selective activators of these enzymes mimicked the effect of FTY720P which was shown by western blot analysis to increase IκB degradation. PGE₂ was found to act via its EP2 receptors, and its effect was still manifested in the presence of calphostin, and the NF-κB inhibitor but disappeared completely in presence of inhibitors of PKA, NOS, and ERK, suggesting that PGE₂ is downstream of PKC and NF-κB and upstream of PKA, NO, and ERK. The effect of PMA, a PKC activator, was completely abolished by NF-κB inhibitor indicating that NF-κB is downstream of PKC and upstream of PGE₂. Furthermore, western blot analysis showed phosphorylation of ERK by dibutyrylcyclic adenosine monophosphate, a PKA activator. This phosphorylation did not appear in presence of PTIO indicating that PKA is upstream of NO. It was concluded that FTY 720-P activates PKC via S1P2 receptors leading to NF-κB activation and PGE₂ generation. PGE₂ acts through its EP2 receptor and activates PKA which in turn activates NOS leading to NO production and ERK activation. ERK then inhibits directly or indirectly the Na⁺/K⁺ ATPase.

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ABBREVIATIONS

AKAP	A kinase anchoring protein
ANOVA	One-way Analysis of Variance
ATP	Adenosine triphosphate
BAEC	Bovine aortic endothelial cells
BSA	Bovine serum albumin
C terminus	Carboxy terminus
Caco-2	Human colon carcinoma cell line
cAMP	Cyclic adenosine monophosphate
cGMP	Cyclic guanosine monophosphate
COX	Cyclooxygenase
DAG	Diacylglycerol
DMEM	Dulbecco's Minimal Essential Medium
DMSO	Dimethyl sulfoxide
EDG receptor	Endothelial differentiation gene receptor
EDTA	Ethylenediamine tetra-acetic acid
eNOS	Endothelial nitric oxide synthase
ERK	Extracellular signal regulated kinase
FBS	Fetal Bovine Serum
FDA	Food and Drug Administration
FTY720	Fingolimod
FTY720P	Fingolimod phosphate
GDP	Guanosinediphosphate
GEF	Guanine nucleotide exchange factor
GPCR	G-protein coupled receptor
HepG2	Human hepatoma cell line
HRP	Horse raddish peroxidase
IgG	Immunoglobulin G
IKK	I κ B kinase
iNOS	Inducible nitric oxide synthase
IP3	Inositol 1,4,5-triphosphate
MAPK	Mitogen-activated protein kinase
MDCK	Madin-Darby canine kidney cells
MEK	MAP/ERK kinase
MTAL	Medullary thick ascending loop of Henle cells
N terminus	Amino terminus
NF- κ B	Nuclear factor kappa
nNOS	Neuronal nitric oxide synthase
NO	Nitric oxide
NSAID	Non-steroidal anti-inflammatory drug
PBS	Phosphate buffer saline
PGE ₂	Prostaglandin E ₂

PGE ₂ S	Prostaglandin E ₂ synthase
PGG ₂	Prostaglandin G ₂
PGH ₂	Prostaglandin H ₂
PI3K	Phosphoinositide 3-kinase
PKA	Protein kinase A
PKC	Protein kinase C
PKG	Protein kinase G
PLA ₂	Phospholipase A ₂
PLD	Phospholipase D
PMA	Phorbol 12-myristate 13-acetate
PS	Penicillin/Streptomycin
PTH	Parathyroid hormone
RACK	Receptor of activated-C kinase
RHD	Rel Homology Domain
S1P	Sphingosine-1-phosphate
SDS	Sodium dodecyl sulphate
SEM	Standard error of the mean
Thr	Threonine
TNF	Tumor Necrosis Factor
Tyr	Tyrosine

CHAPTER I

INTRODUCTION

Hepatocytes are polarized cells which exhibit a basolateral membrane facing the blood sinusoids and a canalicular membrane facing the bile containing canalicular lumen (Howard Evans, 1980). The basal membrane plays a crucial role in bile secretion (Gregory, et al. 1975). In fact, bile flow is stimulated by the secretion of osmotically active components like bile salts and to a lesser extent bicarbonate anions across the apical membrane and into the canaliculi. This is followed by water movement into the canaliculi driven by osmosis. On the other hand, the Na^+/K^+ ATPase found in the basolateral membrane of hepatocytes provides the needed driving force for the secondary transport of bile salts and bicarbonates from the blood to the intracellular medium of hepatocytes through respectively the sodium/bile salt and the sodium/bicarbonate symporters (Esteller, 2008; Boyer, 2013). Thus, the Na^+/K^+ ATPase plays a critical role in bile synthesis and secretion (Simon, et al. 1977; Layden & Boyer, 1976). Therefore, changes in its activity are expected to be accompanied with changes in bile flow.

Sphingosine-1-phosphate (S1P), a sphingolipid mediator known for its versatile functions in mammalian cells, has been shown to down-regulate the Na^+/K^+ ATPase in HepG2 cells (Dakroub & Kreydiyyeh, 2012) which are cells derived from a human cancerous cell line (Mersch-Sundermann, et al. 2004). Therefore, S1P may have many physiological implications on liver functions as a consequence of the reduced activity of the Na^+/K^+ ATPase (Yu, 2003) such as decreasing bile flow and inducing apoptosis, since cell death has always been found to accompany inhibition of the ATPase. Nevertheless, the mediators involved in this inhibitory effect have not been identified yet.

Since S1P is insoluble in water and sparingly soluble in any organic solvent, we opted to use instead its well-recognized agonist fingolimod (FTY720) which has been approved for the treatment of Multiple Sclerosis (Brinkmann, et al., 2010) and is being considered as a treatment for other diseases as well (Zhang, 2013). Side effects of the FTY720 treatment include nausea, diarrhea, and bradycardia. Its effects on liver function have not been well studied although increases in the level of transaminase and other liver enzymes have been demonstrated (Kappos, et al., 2006); an increase in the level of these enzymes is usually correlated with liver damage (Giboney, 2005) and a decrease in the activity of the ATPase is correlated with apoptosis (Yu, 2003).

This work aims at delineating the signaling pathway mediating the effect of S1P on the Na^+/K^+ ATPase using its agonist FTY720P, the biologically active form and the product of FTY720 phosphorylation in vivo. Targeting the different mediators and changing their activities may circumvent any undesirable effects of S1P or FTY720 on liver functions.

CHAPTER II

LITERATURE REVIEW

A. The Na⁺/K⁺ ATPase

1. Properties

The Na⁺/K⁺ ATPase is expressed in all mammalian cells. It plays a key role in the formation of the membrane potential through the transport of three sodium ions to the extracellular medium and two potassium ions to the intracellular medium against their concentration gradients at the expense of one ATP molecule. The established sodium electrogenic pump provides the driving force for the secondary transport of many metabolites (Skou & Esmann, 1992).

The Na⁺/K⁺ ATPase is a member of the family of P-ATPases which also includes the Ca²⁺ ATPase of the plasma membrane and the sarcoplasmic reticulum and the H⁺/K⁺ ATPase of the colon and stomach (Sachs & Munson, 1991). A catalytic cycle that involves the transient formation of a phosphorylated aspartyl residue is what characterizes this family of ATPases (Ohtsubo, et al. 1990). However, of all P-ATPases, only the Na⁺/K⁺ ATPase is sensitive to cardiac glycosides such as Ouabain (Repke, et al. 1996), and unlike the other members which consist of a single subunit, the Na⁺/K⁺ ATPase is a heterooligomer of two subunits: the alpha subunit and the beta subunit (Axelsen & Palmgren, 1998).

The much larger alpha subunit contains the binding site for cardiac glycosides and consists of ten transmembrane domains with both the N and the C termini on the cytoplasmic side. A large cytoplasmic loop found between transmembrane domains 4 and 5 is believed to contain the phosphorylation and the ATP binding sites (Lingrel & Kuntzweiler, 1994). This is why, the alpha subunit is considered as the catalytic subunit (Ohtsubo, et al. 1990). Four isoforms

expressed by four separate genes have been identified for the alpha subunit, characterized each by a distinct tissue and cell distribution (Lingrel & Kuntzweiler, 1994). The α_1 isoform is ubiquitously expressed (Lingrel, et al. 1987). The α_2 subunit is expressed in the brain, adipocytes, and skeletal muscles while the α_3 subunit was mainly detected in the heart, nerves, and the brain, and the α_4 subunit in the testes (Juhaszova & MP, 1997). A high degree of homology was observed among three of the four alpha subunits: alpha1, 2, and 3 (Blanco & Mercer, 1998).

The beta subunit is also crucial for the proper functioning of the enzyme. It protects the alpha subunit from degradation by proteolytic enzymes and acts as a chaperon that allows the proper folding and delivery of the alpha subunit to the plasma membrane (Kawamura & Noguchi, 1991). Structurally, the beta subunit is much different from the alpha subunit. It consists of a single transmembrane domain with small cytoplasmic but large extracellular domain (Kawamura & Noguchi, 1988). The extracellular segment is glycosylated and contains several disulfide bridges allowing the proper interaction with the alpha subunit (Beggah, et al. 1997). Three isoforms for the beta subunit have been identified so far. Beta 1 is known for its ubiquitous expression, Beta 2 is mainly expressed in the brain (Avila, et al. 1998) while Beta 3 is primarily expressed in the lungs, skeletal muscle, in addition to the brain (Appel, et al. 1996). Unlike the alpha subunit isoforms, the beta isoforms do not show a high degree of homology (Kawakami, et al 1986).

The alpha and the beta subunits assemble in a 1:1 ratio (Jaunin, et al. 1993; Jaisser, et al. 1994) in the endoplasmic reticulum soon after they are synthesized (Geering, et al. 1996), and only assembled subunits manage to get transported out of the endoplasmic reticulum (Béguin, et al. 2000; Ackermann & Geering, 1990). Studies have shown that there is no preference among the

different alpha isoforms for a specific beta isoform. The resultant combination of isoforms is tissue specific (Lingrel, et al 1987).

2. Regulation

The expression and the activity of the Na^+/K^+ ATPase can be modulated by a number of kinases cytokines and mediators.

Serine threonine kinases such as PKA, PKC, and PKG have been shown to directly phosphorylate the alpha subunit of the Na^+/K^+ ATPase leading to a decrease or an increase in the activity of the pump (Bertorello & Katz, 1993). These kinases have been also involved in mediating signaling pathways that lead to a modulation in the activity of the Na^+/K^+ ATPase. For instance, Dopamine has been shown to mediate its inhibitory effect on the Na^+/K^+ ATPase via PKA in cells of the cortical collecting duct (Satoh, et al 1992) and via PKC in MDCK cells (Shahedi, et al. 1992).

Moreover, the extracellular-signal regulated protein kinase (ERK) has been reported to mediate the parathyroid hormone (PTH) induced activation of PKC, which is essential for the hormone's inhibitory effect on the Na^+/K^+ ATPase in opossum kidney cells (Khundmiri, et al 2005). ERK 1/2 has been also reported to mediate insulin's and C-peptide's stimulatory effects on the Na^+/K^+ ATPase in human skeletal muscle cells and human renal tubular cells respectively (Zhong, et al. 2004; Al-Khalili, et al. 2004).

Prostaglandin E2 (PGE_2) exerts also an inhibitory effect on the sodium potassium pump in a number of mammalian cells including cells of the inner medullary collecting duct (Jabs, et al. 1989), cells of the cortical collecting duct (Warden & Stokes, 1993), renal tubular epithelial cells (Zeidel, et al. 1989), cells of the non-pigmented layer of ciliary epithelium (Delamere, et al.

1997), HepG2 cells (Kreydiyyeh, et al. 2007), and Madin-Darby canine kidney cells among other cell lines (MDCK cells) (Cohen, et al. 1994). PGE₂ has also been reported to elicit a stimulatory effect on the Na⁺/K⁺ ATPase at low doses (Kreydiyyeh, et al. 2006).

Nitric oxide has also been reported to inhibit the Na⁺/K⁺ ATPase activity in several types of cells including opossum kidney cells (Liang & Knox, 1999), mouse proximal tubule epithelial cells (Guzman, et al. 1995), and bovine ciliary processes (Ellis, et al. 2001). Furthermore, it has also been reported to inhibit the transcription of the alpha subunit of the sodium pump in thick ascending limb of Henle cells, MTAL cells (Kone & Higham, 1999).

B. Sphingosine-1-Phosphate

Sphingosine-1-phosphate (S1P) is a sphingolipid signaling molecule that is currently gaining a great deal of interest in the scientific field (Spiegel & Milstien, 2003). The biosynthesis of this molecule begins with the production of ceramide either through a de novo synthesis in the endoplasmic reticulum (Futerman, et al. 1990) or through the recycling of the sphingomyelin found in the plasma membrane by the action of the enzyme sphingomyelinase (Schmelz, et al. 1994). Ceramide is then changed by ceramidase into sphingosine (Hannun & Obeid, 2008) which will be phosphorylated by the action of one of the sphingosine kinases 1 or 2 yielding sphingosine-1-phosphate (Igarashi & Yatomi, 1998). It is important to note that even though S1P kinases 1 and 2 catalyze the formation of the same product, they possess distinct catalytic properties, temporal expression and tissue distribution (Takabe, et al. 2008).

Sphingosine-1-phosphate may be dephosphorylated by S1P phosphatase back to sphingosine, or it may be broken down irreversibly into hexadecanal and phosphoethanolamine (Maceyka, et al. 2008).

S1P has been reported to act as an intracellular signaling molecule (Van Brocklyn, et al., 1998). However, most of its actions are mediated by what is known as the “Inside Out Mechanism” whereby S1P is secreted to the extracellular medium to act as an autocrine agent on one of its five G-protein coupled receptors (GPCR) namely: S1P1, S1P2, S1P3, S1P4, and S1P5 (Spiegel & Milstien, 2000; Takabe, et al. 2008). S1P receptors are also known as “Endothelial Differentiation Gene” receptors because they were first identified when studying the differentiation process of endothelial cells (Hla & Maciag, 1990). Every S1P receptor can be coupled to one or several types of G-proteins. In fact, S1P1, and S1P5 are coupled to Gi whereas S1P2 and S1P3 are coupled to Gq, G12/13, and Gi. S1P4 is only coupled to Gi and G12,13 (Brinkmann, 2007). Hence, the specific cellular response elicited by S1P depends on the S1P receptor activated and the G-protein coupled to it. This explains how S1P can activate various signaling pathways to mediate a wide range of cellular responses including the regulation of cell growth and motility, and the control of the trafficking of immune cells (Spiegel & Milstien, 2003; Zhang, et al. 1991; Sugimoto, et al. 2003).

1. PGE₂ and S1P

PGE₂ is one of the mediators that are known to participate in S1P-activated signaling pathways. S1P has been reported to activate the COX-2 enzyme in renal mesangial cells via activation of its S1P2 receptors (Völzke, 2014), and induces the production of PGE₂ in L929 fibroblast cells in response to TNF- α (Pettus, et al., 2003). S1P has also been shown to induce PGE₂ production in human tracheal smooth muscle cells via a c-Src dependent mechanism (Hsu, et al. 2014) and in vascular smooth muscle cells via an Akt-ERK1/2-NF- κ B pathway (Hsieh, et al. 2006).

2. *NO and SIP*

Nitric oxide (NO) is also a mediator of SIP-action. In fact, SIP was shown to activate eNOS in bovine aortic endothelial cells (BAEC) via an Akt-dependent pathway (Igarashi, et al. 2001) and to mediate the TNF- α activation of eNOS in human endothelial cells via the same pathway (De Palma, et al. 2006), while in bEnd.3 cells, it induced eNOS activation via both the calcium-dependent and the Akt-dependent pathways (Mulders, et al. 2006).

3. *ERK1/2 and SIP*

Another common mediator of SIP signaling pathways is ERK1/2. SIP activated ERK1/2 in human umbilical vein endothelial cells, human kidney 293 cells, HTC4 rat hepatoma cells, and Cos-1 cells via EDG-1 and EDG-3 receptors (Takeya, et al. 2003). SIP also participated in the regulation of osteoblast differentiation via ERK1/2 (Sato, et al. 2012). Moreover, SIP stimulated phospholipase D and consequently aldosterone secretion by zonaglomerulosa cells (ZG cells) by activating ERK1/2 and PI3K/Akt (Brizuela, et al. 2007).

C. *FTY720 and FTY720-Phosphate*

Fingolimod (FTY720) which is derived from a structural modification of the fungal metabolite, myriocin (Fujita, et al., 1994), has been approved by the FDA as an oral treatment for Multiple Sclerosis (Brinkmann, et al., 2010), and its use as a potential treatment for a range of other diseases is currently being considered as well (Zhang, et al. 2013). However, it is only after the phosphorylation of FTY720 in vivo by the enzyme sphingosine kinase 2, that the prodrug, FTY720, is transformed into the biologically active molecule, FTY720-phosphate (Zemann, et

al., 2006). Given that the enzyme sphingosine kinase 2 is present abundantly in the liver, it has been proposed that the phosphorylation of FTY720 takes place mainly in the liver (Liu, et al., 2000). However, it has been shown later on, that this phosphorylation can also take place in platelets that release the phosphorylated FTY720 into the blood in a stimulus-independent manner. Moreover, it is important to note that FTY720 undergoes a continuous cycle of phosphorylation and dephosphorylation in vivo (Anada, et al. 2007). FTY720P can be dephosphorylated extracellularly by the action of ectophosphatases (Kai, et al. 1997). FTY720-phosphate is a very well-known agonist for sphingosine-1-phosphate and acts by activating one of the sphingosine-1-phosphate receptors (Takabe, et al. 2008). For instance, its role in the treatment of Multiple Sclerosis is mediated via S1P1 receptors expressed by thymocytes and lymphocytes. The trafficking of those cells into the bloodstream is controlled by a stimulus elicited by the S1P1 receptor (Gräler & Goetzl, 2004). By binding to the S1P1 receptor, FTY720-phosphate triggers the internalization and the subsequent degradation of the receptor, leading to its down-regulation in the long run. Hence, it is depriving lymphocytes and thymocytes of the signal triggering their movement into the blood, and it is therefore causing a state of immunosuppression that could relieve the symptoms of an autoimmune disease such as Multiple Sclerosis (Matloubian, et al., 2004).

D. Heterotrimeric G-proteins

Heterotrimeric guanine nucleotide binding proteins which are commonly known as heterotrimeric G-proteins are signaling molecules that contribute to the propagation of the signal elicited by many heptahelical receptors. Heterotrimeric G-proteins are composed of an alpha, beta, and a gamma subunit (Dhanasekaran & Gutkind, 2001). The beta and the gamma subunits

form a membrane localized heterodimer, and the membrane localization of the alpha subunit and its association with the GPCR are enhanced through its association with the beta-gamma heterodimer (Evanko, et al. 2001; Robillard, et al. 2000). Moreover, the alpha subunit alternates between an inactive GDP bound state and an active GTP bound state. The beta-gamma heterodimer decelerates the rate of release of the GDP bound to the alpha subunit. Therefore, the alpha subunit exists in an inactive state when it is bound to the beta-gamma heterodimer (Brandt & Ross, 1985). It is only when a ligand binds to the GPCR that the activation of the alpha subunit occurs: a conformational change occurs in the receptor transforming the alpha subunit into a guanine nucleotide exchange factor (GEF) that favors the release of GDP and its replacement with GTP which is abundantly found in the intracellular medium, activating thus the alpha subunit which dissociates from the beta-gamma heterodimer and acts on downstream effectors (Wall, et al. 1998). This activation is however short lasted because of the intrinsic GTPase activity of the alpha subunit which hydrolyzes GTP back to GDP and induces the re-association of the alpha subunit with the beta-gamma heterodimer (Ford, et al., 1998).

Four classes have been defined for the alpha subunit of heterotrimeric G-proteins, and cellular targets have been identified for each one of these classes (Simon, et al. 1991). The $G_{\alpha s}$ subunit activates the enzyme adenylyl cyclase leading to the production of cAMP and the subsequent activation of protein kinase A (PKA) (Ross & Gilman, 1977). The $G_{\alpha i}$ has an opposite effect: it inhibits adenylyl cyclase and consequently the production of cAMP (Hildebrandt, et al. 1983). The $G_{\alpha q}$ subunit activates the enzyme phospholipase C which is responsible for the production of diacylglycerol (DAG) and inositol 1, 4, 5-triphosphate (IP3) as a result of the hydrolysis of phosphoinositol 4, 5-bisphosphate (PIP2) abundantly present in the plasma membrane (Rhee, 2001). DAG activates protein kinase C (PKC) directly while IP3 activates PKC indirectly by

triggering an increase in intracellular calcium concentrations (Mochly-Rosen, et al. 2012).

Finally, $G\alpha_{12/13}$ is known to activate the monomeric GTPase RhoA which in turn activates the Rho kinase signaling pathway (Worthylake, et al. 2000).

On the other hand, five classes of the beta subunit and twelve classes of the gamma subunits have been identified resulting in a huge number of possible combinations for the formation of the beta-gamma heterodimers (Simon, et al. 1991). Moreover, the beta-gamma heterodimer activates on its own many effectors like the inward-rectifier K^+ channel (Logothetis, et al. 1987) and the calcium channels (Garcia, et al., 1998).

E. Protein Kinase C

Protein Kinase C (PKC) is a family of serine-threonine kinases that phosphorylate a large number of protein substrates. This family of kinases is mainly comprised of eight isozymes namely PKC α , β I, β II, γ , δ , ϵ , θ , and η . These isoforms are encoded by seven related genes and are further divided into either conventional PKCs or novel PKCs. Moreover, it was demonstrated that all these isozymes are expressed ubiquitously in all tissues and could be rendered active by the same stimuli to mediate different cellular responses (Coussens, et al. 1986; Ono, et al. 1988).

All PKC isozymes are composed of a carboxy-terminal catalytic region and an amino-terminal regulatory region. In the inactive state, the regulatory region remains bound to the catalytic region which contains the ATP binding site, the magnesium binding site, and the substrate specific binding site of the enzyme. The dissociation of this intramolecular interaction between the two regions cancels the inhibitory effect of the regulatory region and leads to the activation of PKC (Mochly-Rosen, et al. 2012).

In fact, the activation of PKC can be triggered by hormones, growth factors, or neurotransmitters which activate signaling pathways involving the enzyme phospholipase C which catalyzes the production of diacylglycerol (DAG) and inositol 1,4,5-triphosphate (Rhee, 2001; Mochly-Rosen, et al. 2012). Diacylglycerol binds to the C1 domain of the regulatory region of PKC, and this binding is needed for the activation of all the isozymes of PKC. DAG, alone, is sufficient to activate novel PKCs. However, conventional PKCs require the binding of calcium to the C2 domain of the regulatory region in addition to the binding of DAG to the C1 domain of the same region for activation (Bollag, 2009).

After activation, PKC gets translocated from the cytosol to the plasma membrane or other subcellular sites that can vary in an isozyme-specific manner, and binds at these sites to specific anchoring proteins known as Receptor of Activated-C Kinase (RACK) proteins (Mochly Rosen, 1995). The binding of DAG, calcium, and RACK to PKC render it ready to phosphorylate its specific substrates leading to various cellular responses (Mochly-Rosen, et al. 2012).

F. Protein Kinase A

Protein kinase A (PKA) is the key mediator of almost all cellular processes that are dependent on cyclic adenosine monophosphate (cAMP). In mammalian cells, the PKA holoenzyme is composed of two regulatory subunits and two catalytic subunits, and remains inactive until two cAMP molecules bind to each regulatory subunit (Singh, et al., 1998) and cause their dissociation from the catalytic subunits rendering them active and ready to phosphorylate different cytoplasmic or nuclear substrates on serine-threonine residues (Skalhegg & Tasken, 2000).

Three isoforms have been identified for the catalytic subunit, and these include $C\alpha$, $C\beta$, and $C\gamma$ while the regulatory subunit can exist in four different isoforms. These include, on one hand, isoforms $RI\alpha$ and $RI\beta$ present in the PKA-I isoform, and isoforms $RII\alpha$, and $RII\beta$ which are mainly found in the PKA-II isoform (Sapio, et al., 2014). The RI isoforms have a higher affinity for cAMP than the RII isoforms. As a result, the PKA-I isoforms are more sensitive to cAMP than the PKA-II isoforms and could be activated at lower cAMP concentrations. Moreover, the type I isoforms of PKA have cytoplasmic localizations while the type II isoforms are anchored to subcellular organelles (Skalhegg & Tasken, 2000).

Finally, even though PKA has been identified as a mediator in various signaling pathways that control a wide range of cellular responses, it is a highly specific signaling molecule, and this specificity cannot be only attributed to the types of regulatory or catalytic isoforms expressed but rather to the subcellular localization of PKA achieved by the binding of the regulatory subunits to anchoring proteins known as A kinase anchoring proteins (AKAP) (Pidoux & Taskén, 2010).

G. NF- κ B

NF- κ B is a transcription factor with numerous biological roles in both health and disease states. This family of transcription factors includes five members (Rel A, Rel B, p52/p100, c-Rel, and p50/105) which can normally exist as hetero- or homo-dimers. In its inactive state, NF- κ B is always bound to I κ B proteins through its Rel Homology Domain (RHD) which is also implicated in the dimerization process and the binding to the DNA (Hayden & Ghosh, 2004). I κ B proteins prevent the translocation of the NF- κ B/I κ B complex to the nucleus therefore rendering this transcription factor inactive. Phosphorylation of I κ B by I κ B kinase (IKK), an enzyme complex consisting of an alpha subunit (IK α), a beta subunit (IK β), and a regulatory subunit, leads to its

polyubiquitination and degradation by the ubiquitin-proteasome system. This allows the translocation of NF- κ B to the nucleus to bind to specific promoter regions in the target genes (Karin & Ben Neriah, 2000).

Activation of NF- κ B can occur via a classical pathway or an alternative pathway. The classical pathway involves the activation of the beta subunit of IKK while the alternative pathway involves the activation of the alpha subunit of IKK (Bonizzi & Karin, 2004).

H. Prostaglandin E₂ (PGE₂)

The versatile lipid mediator, prostaglandin E₂, is a prostanoid with numerous pro-inflammatory as well as anti-inflammatory actions (Honda, et al. 2006; Kunikata, et al. 2005). It is produced in abundance in the human body and across different animal species (Reader, et al. 2011). It is derived from the polyunsaturated fatty acid, arachidonic acid, which is released from the plasma membrane by the action of the enzyme phospholipase A₂ (Funk, 2001). Cyclooxygenase (COX) enzymes then act upon this fatty acid in two sequential steps whereby arachidonic acid undergoes cyclization to produce PGG₂ followed by reduction to produce PGH₂ (Smith, et al. 2000). Various synthases then act upon PGH₂ giving rise to different prostanoids including PGE₂ which is produced by the action of the enzyme prostaglandin E₂ synthase (PGE₂S) (Samuelsson, et al. 2007).

Soon after its production, PGE₂ is released into the extracellular medium and acts in an autocrine or paracrine way by activating one of its four G-protein coupled receptors namely, EP1, EP2, EP3, and EP4 (Narumiya, et al. 1999). The EP3 and the EP4 receptors are ubiquitously distributed among tissues while the EP1 and the EP2 receptors are less widely distributed with the EP2 receptor having the most restricted distribution among the four receptors

(Kutsuyama, et al. 1995). Moreover, these four receptors are known to activate distinct signaling pathways. The EP1 receptor triggers an increase in cytosolic calcium concentrations that is unrelated to phosphoinositides and is solely dependent on extracellular calcium. Therefore, this GPCR is believed to regulate calcium channels through an unidentified G-protein (Tabata, et al. 2002). On the other hand, the EP2 and the EP4 receptors are coupled to Gs proteins and increase the production of cAMP (Li, et al. 2000; Tomita, et al. 2002), while EP3 is coupled to Gi and inhibits the production of cAMP (Sugimoto, et al., 1992).

As mentioned above, COX enzymes are key enzymes in the production of PGE₂. Two isoforms of this enzyme have been defined namely, COX-1 and COX-2. The COX-1 isoform is a constitutively active enzyme while the expression of the COX-2 isoform is usually induced by multiple cytokines and growth factors. This could be attributed to differences in the amino acid sequences at the promoter regions of each of these two isoforms (Tanabe & Tohnai, 2002; Morita, 2002). Moreover, even though both isoforms act upon arachidonic acid and carry out the sequential cyclization and reduction reactions, each isoform has a preference for a specific pool of arachidonic acid found in the cell. COX-2 acts on endogenous arachidonic acid which is usually found at low intracellular concentrations while COX-1 acts on the arachidonic acid which is derived from extracellular sources or released during cellular injury or acute inflammation and which is present usually at higher intracellular concentrations (Shitashige, et al. 1998). Finally, even though PGE₂ synthase provides a promising therapeutic target for the control of the production of PGE₂, no specific pharmacological inhibitors have been yet identified for this enzyme. Both isoforms of the COX enzyme can be inhibited by non-steroidal anti-inflammatory drugs (NSAIDs), and selective inhibitors of the COX-2 enzyme which

decrease the production of PGE₂ without eliciting the gastro-toxic side effects of inhibiting the COX-1 isoform also exist (Park, et al. 2006; Samuelsson, et al. 2007).

1. NF-κB and COX

NF-κB has been reported to stimulate the expression of the inducible COX enzyme, COX-2. The Interleukin-1 beta induced COX-2 expression (Lee, et al., 2004), and the TNF-α induced up-regulation of COX-2 in synovial fibroblasts (Ke, et al., 2007) were both found to be mediated by NF-κB. In endometrial cancer cells, NF-κB up-regulates COX-2 expression via an Akt dependent pathway (St Germain, et al. 2004). COX-2 has also been shown to be up-regulated by C-peptide in Swiss 3T3 fibroblasts in a PKC/NF-κB pathway (Kitazawa, et al. 2006).

2. PKC and COX

Up-regulation of the COX-2 enzyme can be also induced by PKC. In fact, it has been shown that in non-pigmented ciliary epithelial cells the inhibition of the Na⁺/K⁺ ATPase by PGE₂ was dependent on the activation of COX-2 by PKC (Delamere, et al. 1997). Moreover, PKC-α was shown to modulate COX-2 expression in response to LPS or IFN-γ in RAW264.7 cells (Giroux & Descoteaux, 2000). Studies conducted on mouse keratinocytes demonstrated also a stimulatory effect of PKC-α on the COX-2 enzyme (Wang, et al. 2001).

I. Nitric Oxide (NO)

Nitric oxide (NO) is a signaling molecule with diverse physiological functions in various mammalian systems such as the nervous system, the immune system, and the cardiovascular system. However, NO exerts pathophysiological effects when produced in excessive amounts

and contributes to various disease states such as chronic inflammation, arthritis, strokes, diabetes, and neurodegenerative diseases (Nathan, 1992; Gross & Wolin, 1995).

Nitric oxide is synthesized by a family of enzymes known as Nitric Oxide Synthases (NOS). Three isoforms of NOS have been identified, and these include the inducible NOS (iNOS), the endothelial NOS (eNOS), and the neuronal NOS (nNOS). The name of the iNOS isoform originates from the fact that it was first reported in immunoactivated macrophages which did not express this enzyme in the inactive state. However, it was later reported that the iNOS isoform can be constitutively active under normal physiological conditions in certain cells (Guo, et al. 1995). Similarly the nNOS and the eNOS isoforms have been named as such because they were first described in neuronal and endothelial tissues respectively. However, later reports have revealed a much wider tissue distribution of those two isoforms which were first believed to be constitutively expressed in tissues, but found later on to be inducible in a number of cells (Michel & Feron, 1997).

The three NOS isoforms catalyze the oxidation of L-arginine into L-citrulline, a reaction which liberates nitric oxide and occurs in the presence of the co-substrates oxygen and NADPH and the cofactors FAD, FMN, and tetrahydrobiopterin. Moreover, this reaction also requires the binding of all three isoforms to calmodulin, but only the eNOS and the nNOS isoforms require high intracellular calcium concentrations while the iNOS isoform can carry out the reaction even at low intracellular calcium concentrations (Nathan & Xie, 1994).

After its synthesis, NO can exert its effect within the same cell in which it was synthesized or it can diffuse to the extracellular medium to act as a signaling molecule on neighboring cells (Ignarro, 1990). In fact, being small in size and hydrophobic, nitric oxide readily diffuses across the cell membrane and acts in a concentration dependent manner (Persson, et al. 1994). Unlike

other signaling molecules which mediate their actions by activating cell surface or nuclear receptors, NO elicits its actions by directly interacting with its targets through various chemical reactions (Gross & Wolin, 1995). In fact, one of the well established actions of NO is the nitrosation of the heme prosthetic group of the soluble guanylylcyclase enzyme triggering its activation (Ignarro, 1990). This NO intracellular receptor mediates the NO induced cellular responses by the production of cGMP which in turn activates the cGMP dependent protein kinase (PKG) in addition to the cGMP gated ion channels and the cGMP regulated phosphodiesterases. Various responses in the cardiovascular and the nervous system ranging from the inhibition of platelet aggregation and smooth muscle relaxation to the regulation of synaptic transmission have been linked to this NO-cGMP signaling cascade (Friebe & Koesling, 2003).

NO has also been reported to directly activate the cyclooxygenase enzymes through a mechanism analogous to the nitrosation of the heme group of guanylylcyclase (Salvemini, et al. 1993). However, instances of an inhibitory effect of NO on prostaglandin production in chondrocytes and in activated macrophages have also been reported (Amin, et al. 1997; Habib, et al. 1997), and it has been demonstrated that NO can exert distinct effects on COX-1 and COX-2 stimulating the former and inhibiting the latter (Clancy, et al., 2000).

The GTPase activity of many enzymes including the p21 ras enzyme can also be activated by NO (Lander, et al. 1993) while enzymes such as thromboxane synthetase, peroxidase, catalase, and lipooxygenases can be inhibited by this signaling mediator (Gross & Wolin, 1995).

The production of NO by NOS enzymes can be regulated at many levels. The availability of the needed co-substrates and co-factors for the production of NO represents one of the factors controlling NOS activity. Moreover, several serine/threonine kinases have been reported to

phosphorylate the three NOS isoforms and evidence suggesting an alteration in NOS activity as a result of these modifications has been reported (Nathan, 1992). For instance, phosphorylation of eNOS by PKA, PKG, and Akt has been reported to increase the activity of eNOS (Zhang, et al. 2009; Butt, et al. 2000). Moreover, studies have shown that the direct phosphorylation of eNOS by the Akt through the Akt/ PI3K pathway has been proposed as a calcium-independent route of eNOS activation since such phosphorylation of eNOS by Akt activated this enzyme even at sub-optimal intracellular calcium levels (Dimmeler, et al. 1999). PKA and PKG have also been shown to activate eNOS in a calcium dependent and independent way (Butt, et al., 2000). On the other hand the expression of the iNOS can also be induced by cytokines and bacterial products that up-regulate the iNOS genes by means of several transcription factor such as NF- κ B (Schreck, et al 1992).

1. NO and PGE₂

As previously noted, NO is a regulator of COX-2 activity and PGE₂ is an activator of NOS and consequently the production of NO. EP4 receptors mediated the PGE₂ activation of iNOS in murine breast cancer cells (Timoshenko, et al. 2004), and eNOS in aortic rings causing blood vessel (Hristovska, et al., 2007). Moreover, endogenous PGE₂ has also been reported to up-regulate iNOS in microglial cells (Minghetti, et al. 1997).

J. Extracellular signal regulated kinase 1 and 2 (ERK1/2)

The extracellular signal regulated kinase 1 and 2 (ERK1/2) cascade is one of the four mitogen-activated protein kinase (MAPK) signaling pathways that provide the link between membrane receptors and intracellular cytoplasmic or nuclear targets. The other three cascades include the c-

jun 1 and 3 pathway, the p38 MAPK pathway, and the ERK5 pathway. While the ERK1/2 pathway mediates mitogenic signals, and the c-jun and the p38 MAPK pathways mediate signals that are related to stress, the ERK5 pathway seems to mediate both mitogenic and stress-related signals (Keshet & Seger, 2010). All four MAPK pathways are activated by a GTP-binding protein which in turn activates the enzyme MAPK kinase kinase (MAP3K). This enzyme further activates the MAPK kinase enzyme which phosphorylates the MAPK enzyme and activates it. While the enzymes, MAP3K and MAPK kinase, participate merely in the relay of the signals in the activated cascades, the active MAPK enzyme is responsible for the direct phosphorylation of the different substrates that are targeted by the MAPK signaling pathways in order to elicit various cellular responses such as the control of differentiation, proliferation, survival, and even apoptosis (Raman, et al. 2007).

The ERK 1/2 cascade follows the same general model of activation described above. The activation of this pathway begins with an activation of plasma membrane receptors which in turn recruit adaptor molecules such as Grb2 and guanine nucleotide exchange factors such as SOS in order to activate members of the monomeric G-protein family like Ras (Naor, et al. 2000; Kyriakis, et al. 2004; Wellbrock, et al. 2004). This GTP bound protein then activates the Raf enzymes, a family of MAP3K, which in turn activate a type of MAPK kinases known as the MEK through a serine phosphorylation (Kyriakis, et al. 1992; Alessi, et al. 1994). The two known isoforms of MEK, MEK 1 and MEK2, are known to be highly specific for a single substrate which is the MAPK enzyme, ERK (Dhanasekaran & Premkumar, 1998). Full activation of the ERK enzyme is elicited by the phosphorylation of MEK on threonine and tyrosine residues (Payne, et al., 1991). The two isoforms of the ERK enzyme, ERK 1 with a size of 44 kDA and ERK2 with a size of 42 kDA, are highly homologous to the extent that they carry out

analogous functions and undergo similar modes of regulation during most cellular responses (Voisin, et al. 2010). It is important to note that all members of the ERK1/2 cascade have cytoplasmic localizations in the resting state due to several interactions with anchoring proteins (Chuderland & Seger, 2005). However, upon activation, the Raf enzymes get translocated to the plasma membrane or any other intracellular membrane while the MEK and ERK become released to act upon their targets in the cytoplasm or in the nucleus (Yao & Seger, 2009).

1. NO and ERK 1/2

Nitric oxide can target ERK and increase its activity. In fact, NO mediated hypoxia induced phosphorylation and activation of ERK 1/2 in neuronal piglet nuclei (Mishra, et al. 2004). Moreover, an NO/PKG pathway has been reported to be implicated in the activation of ERK 1/2 in purkinje cells (Endo & Launey, 2003). The neurotoxicity associated with NO in glial cells of primary midbrain cultures was also associated with an activation of the ERK 1/2 signaling pathway upon GSH depletion (Canals, et al. 2003).

CHAPTER III

MATERIALS AND METHODS

A. Materials

FTY720P (FTY720P), Glyco-SNAP1, NF κ B inhibitor, Carboxy-PTIO, Western Blotting Luminol Reagent, anti-EDG-1 rabbit polyclonal antibody, anti-EDG-5 rabbit polyclonal antibody, anti-EDG-3 rabbit polyclonal antibody, anti-EDG-6 rabbit polyclonal antibody, anti-EDG-8 rabbit polyclonal antibody, goat anti-rabbit horse raddish peroxidase (HRP) conjugated IgG, and goat anti-mouse horse raddish peroxidase (HRP) conjugated IgG were purchased from Santa Cruz Biotechnology, CA, USA.

Anti-I κ B α mouse monoclonal antibody was purchased from R&D Systems, Minneapolis, MN, USA.

Anti-ERK 1/2 rabbit polyclonal antibody was purchased from Promega, WI, USA.

Anti-p-ERK 1/2 rabbit monoclonal antibody was purchased from Cell Signaling, MA, USA.

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

Phorbol-12-myrsitate-13-acetate (PMA), Adenosine-3',5'-cyclic MonophosphorothioateRp-Isomer Triethylammonium salt (R ρ cAMP), Calphostin C, and PD98059 were purchased from Calbiochem, San Diego, USA.

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

Biorad assay protein reagent and nitrocellulose membranes were purchased from Biorad, California, USA.

Prostaglandin E₂ (PGE₂), Ouabain, Indomethacin, Dulbecco's Minimal Essential Medium (DMEM) with 4500 mg/L Glucose and pyridoxine HCl, Trypsin-EDTA,

Penicillin/Streptomycin, Fetal Bovine Serum (FBS), 10x Phosphate Buffered Saline (PBS) without magnesium and calcium, and Adenosine 5'-triphosphate disodium salt (ATP), 2'-O-Dibutyryl adenosine 3',5'-cyclic monophosphate sodium salt (dbcAMP) were purchased from Sigma, Chemical Co, St Louis Missouri, USA.

The human liver carcinoma cell line, HepG2, 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 HepG2 cells

HepG2 cells at passages 25-45 were grown in DMEM supplemented with 1% penicillin (100µg/ml) and streptomycin (100µg/ml) and 20% FBS at a density of 120,000 cells/ml on 100 mm culture plates. The cells were kept in a humidified incubator (95% O₂, 5% CO₂) at 37°C and treated at 85-90% confluence after an overnight starvation.

2. Treatment of HepG2 cells

a. Dose response study on the effect of FTY720P on the Na⁺/K⁺ ATPase activity

Following starvation, HepG2 cells were treated for 15 minutes with different doses of FTY720P ranging from 0.0075nM to 150nM. An equal volume of the carrier DMSO, was added to the control. The cells were then washed twice with PBS buffer (pH 7.4) scraped in lysis buffer to which protease inhibitors were added (9.9 ml of 150mM histidine buffer pH7.4, 400 µl protease inhibitor (1 tablet/2ml H₂O), 100 µl Triton-X (1mg/mlH₂O), and homogenized thereafter in a polytron at 20,000-22,000 rpm. Proteins in the resulting cell

lysates were quantified colorimetrically at a wavelength of 595nm using the Bradford assay. .
The activity of the Na⁺/K⁺ ATPase was assayed as described below.

b. Determination of the S1P receptor activated by FTY720P

In order to determine the type of S1P receptors activated by FTY720P , S1P2 and S1P3 receptors were blocked respectively with JTE-013 (1μM in DMSO) and CAY10444 (17.4μM in DMF). The blockers were added to the cells 15 min prior to FTY720P.Treatment with FTY720P was as usual for 15 min, at the end of which cells were scraped lyzed and assayed for their ATPase activity.

HepG2 cells were also treated with JTE-013 for 15 min prior to the treatment with CAY10444.

c. Involvement of PKA

To determine if FTY720P acts via PKA, the kinase was inhibited or activated by pre-treating the cells with RpcAMP (PKA inhibitor: 30μM in water), or dbcAMP (PKA activator: 10μM in water) for 15 min. Similarly the involvement of PKC was studied by the use of Calphostin C (PKC inhibitor: 50nM in DMSO) and PMA, a PKC activator (100nM in DMSO).

d. Involvement of PGE₂ and determination of the EP receptor involved

The involvement of PGE₂ was determined by treating the cells 15 min prior to FTY720P with Indomethacin, a COX inhibitor (100μM, DMSO) and directly with exogenous PGE₂ (1nM in water, 15 min).

Since the EP2 receptors were suspected to be the ones mediating the effect of FTY720P on the Na⁺/K⁺ ATPase, the effect of Butaprost, an EP2 agonist, (4μM in DMSO; 15 min) on the pump was studied, to see if it could mimic the effect of FTY720P.

e. Involvement of NF-κB and ERK

The involvement of NF-κB and ERK was studied by assaying for the activity of the Na⁺/K⁺ ATPase in HepG2 cells treated 15 min prior to FTY720P with NF-κB inhibitor (10μg/ml in water) or PD98059 (MEK1/2 inhibitor: 50μM).

Changes in the protein expression of IKB and phospho-ERK were determined also in these cells by western blot analysis, the details of which are described below.

f. Testing for the involvement of NO

To determine if NO is a mediator, the ATPase activity was assayed in cells treated 15 min prior to FTY720P, with PTIO (NOS inhibitor: 30μM in water) or with SNAP1 (NOS activator: 2μM in water ; 15 min)

g. Involvement of intracellular calcium

To test whether calcium is a second messenger in the effect of FTY720P on the pump, HepG2 cells were treated with the calcium chelator, BAPTA-AM (20nM in DMSO) for 15 min prior to the treatment with FTY720P.

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

To locate PKC, PKA, NO, ERK and NF-KB with respect to PGE2, HepG2 cells were treated respectively with PMA, a PKC activator, in presence of indomethacin, an inhibitor of PGE2 synthesis or with PGE2 in presence of RpCAMPA, PTIO PD98059, or NF-KB inhibitor, respective inhibitors of PKA NOS, ERK and NF-κB.

3. *The Na⁺/K⁺ ATPase Activity Assay*

Following the different above treatments, the cells were scraped, homogenized and their proteins quantified. Cell homogenates were diluted up to a concentration of 0.5µg/µl using a histidine buffer (pH 7.4, 150mM). A volume of 13µl of a phosphatase inhibitor cocktail (100µl of pyrophosphate (200mM), 100µl glycerophosphate (200mM), and 800µl of water) and 17µl of 1% saponin were added to 65µl of cell homogenate, and the samples were incubated for 30 min at room temperature. Aliquots of 12µl were then withdrawn from every sample and added to a mixture containing: NaCl (1240mM, 10µl), KCl (200mM, 10µl), MgCl₂ (40mM, 10µl), ATP (30mM, 10µl), and 20µl of histidine buffer. The samples were then incubated for 30 min at 37°C in the presence and absence of ouabain (15mM, 30µl). Ouabain when absent was replaced with 30 µl water. The reaction was stopped using 10µl of 50% Trichloroacetic acid.

The samples were then spun at a speed of 14000rpm for 5 min. A volume of 100µl was withdrawn from the supernatant and mixed with 80µl of Ferrous sulfate-molybdate reagent (0.5g of Ferrous sulfate, 1ml of ammonium molybdate (0.1g/L of 10N H₂SO₄), 9ml of water). The blue color developed correlates with the amount of inorganic phosphate liberated which was measured in a microplate reader at a wavelength of 750nm.

4. Western Blot Analysis

Equal amounts of protein (40 µg) were resolved on 10% SDS polyacrylamide gels, and then transferred to nitrocellulose membranes. These membranes were then blocked for 40 min with blocking buffer (1L of blocking buffer is composed of 1L of 1x PBS, 1ml of Tween20, and 30g of skimmed milk) and then incubated overnight at 4°C with one of the following primary antibodies: anti-p-ERK1/2, anti-ERK1/2, anti-IKB, anti-EDG1, anti-EDG5, anti-EDG3, anti-EDG-6, or anti-EDG8. Equal loading was validated by checking for GAPDH expression using the primary anti-GAPDH antibody. Following incubation with the respective primary antibodies, the membranes were washed with washing buffer and incubated with the secondary antibodies for 2hrs at room temperature. The secondary antibodies include either goat anti-mouse HRP conjugated IgG (for anti-IKB) or goat anti-rabbit HRP conjugated IgG (for all other mentioned antibodies). The signal was detected by chemiluminescence using luminol reagent.

5. Statistical Analysis

The one-way analysis of variance (ANOVA) was used in order to test for statistical significance followed by the Tukey-Kramer multiple comparison test using Excel and Instat software. The results are reported as mean ± SEM.

CHAPTER IV

RESULTS

A. Dose response study on the effect of FTY720P on the Na⁺/K⁺ ATPase in HepG2 cells

HepG2 cells were treated with different doses of FTY720P for 15 min. A significant decrease in the activity of the Na⁺/K⁺ ATPase was observed at concentrations of 7.5 and 15nM by about 48% and 42% respectively (Figure 1). Since a dose of 7.5nM gave a slightly higher inhibition, it was used in all subsequent experiments.

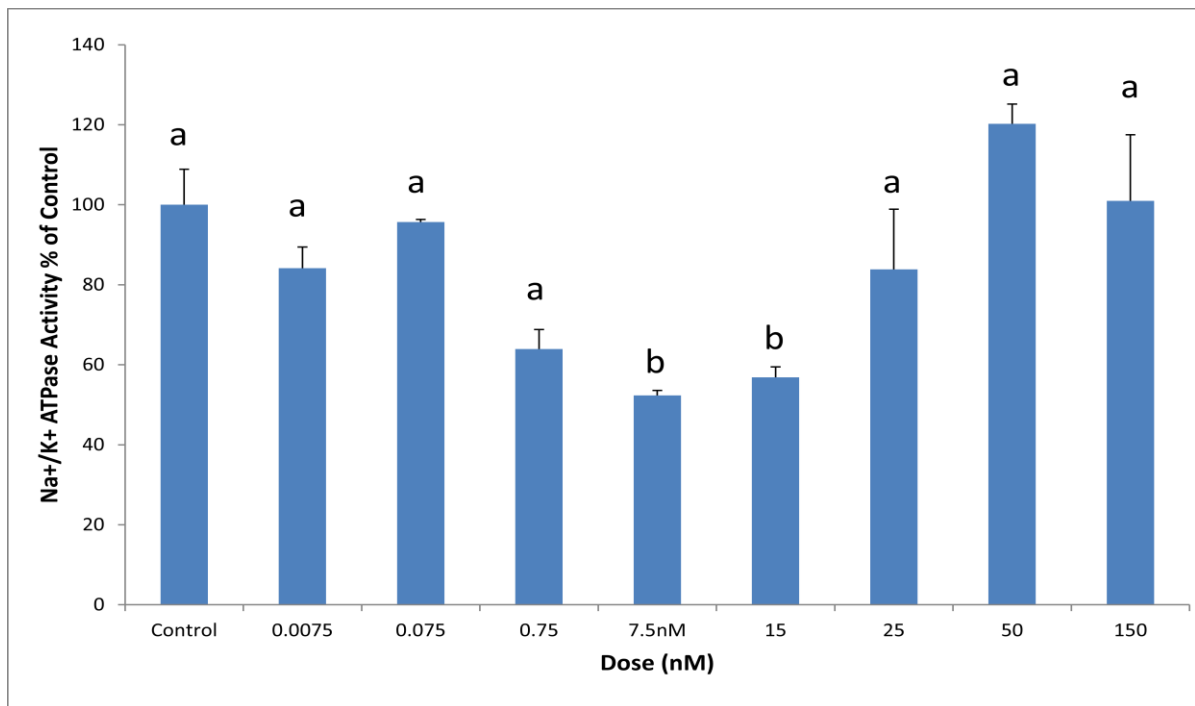


Figure 1: Dose response analysis of FTY720P conducted on HepG2 cells following 15 min treatments. Values are means \pm SEM. N=25. Bars not sharing the same letter are considered significantly different from one another at $p < 0.05$ according to the Tukey-Kramer multiple comparison test.

B. Determination of the S1P receptor activated by FTY720P

Since FTY720P is known to mediate its effects through five different types of S1P receptors, the expression of those receptors in HepG2 cells was studied. Western blot analysis revealed that the five S1P receptors were expressed in HepG2 cells. (Figure2)

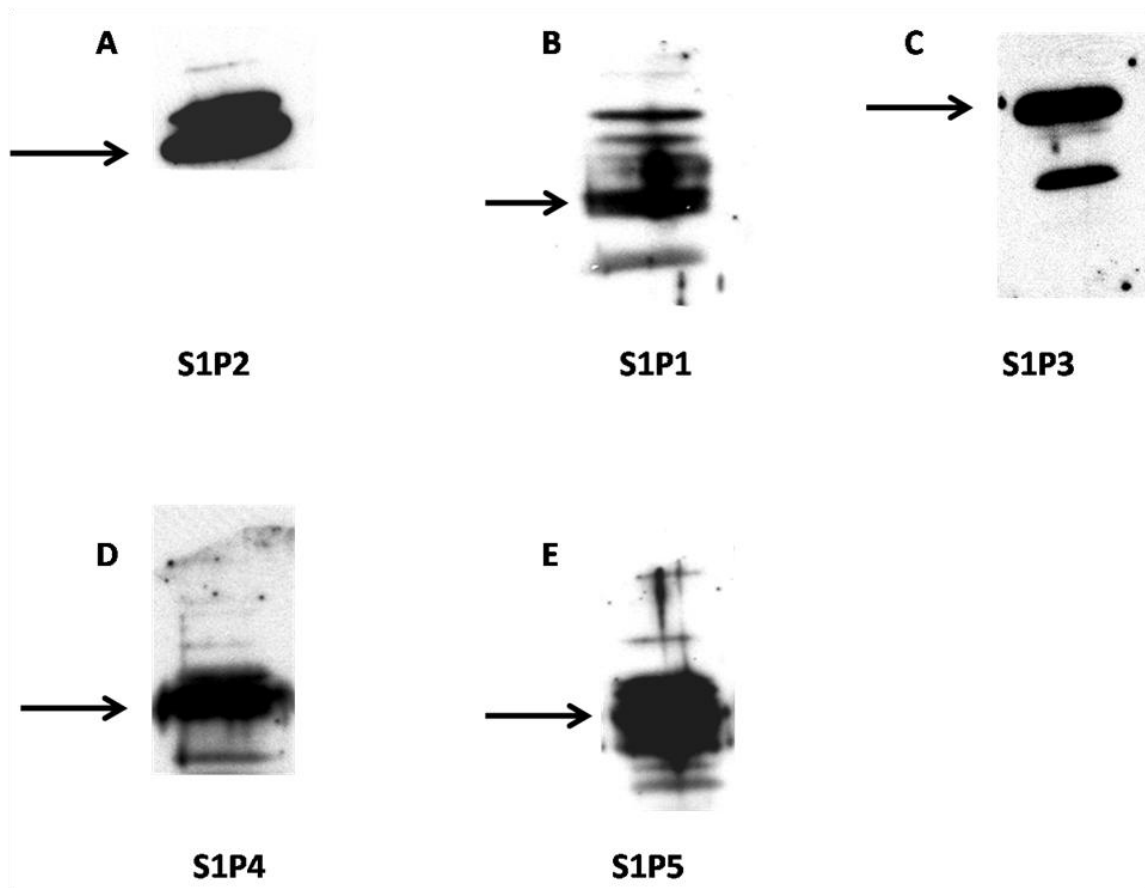


Figure 2: Western blot analysis showing the expression of all five S1P receptors in HepG2 cells A. S1P2. B. S1P1. C. S1P3. D. S1P4 E. S1P5. (protein loaded: 40 μ g).

In order to identify the receptor mediating the effects of FTY720P, blockers of S1P receptors were used. The inhibitory effect of FTY720P disappeared completely in the presence of JTE-013, a selective S1P2 receptor blocker (figure 3).

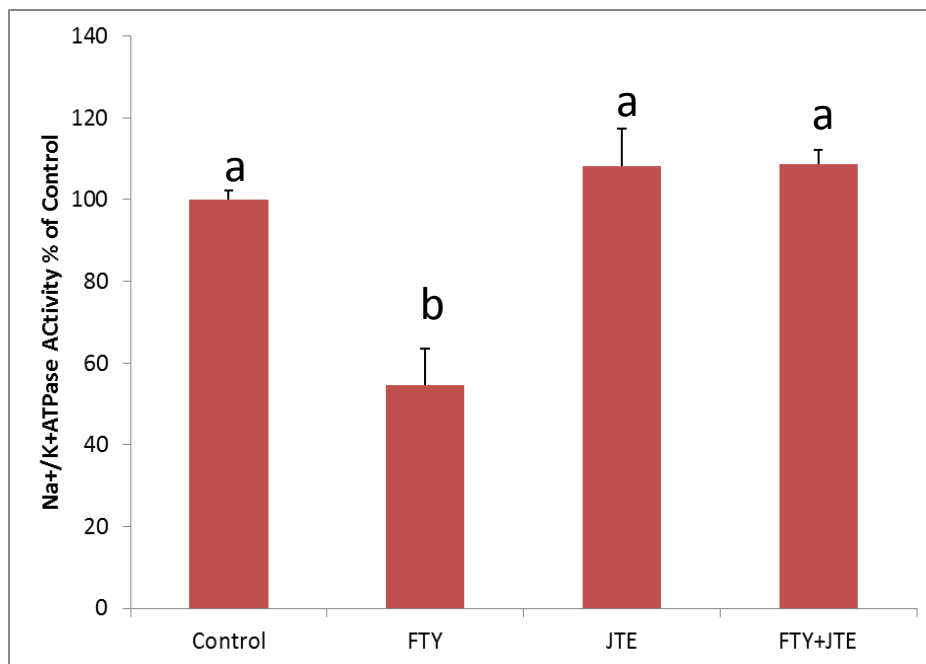


Figure 3: JTE-013 completely abolishes the FTY720P induced inhibition of the Na⁺/K⁺ ATPase in HepG2 cells. Values are means \pm SEM. N=3. Bars not sharing the same letter are considered significantly different from one another at $p < 0.01$ according to the Tukey-Kramer multiple comparison test.

The effect of FTY720P phosphate was mimicked by the S1P3 blocker, CAY10444 (Figure 4a), and in its presence the inhibitory effect of FTY720P was maintained unaltered (Figure 4b).

However, the effect of CAY 10444, disappeared when the cells were pre-treated with JTE-013.

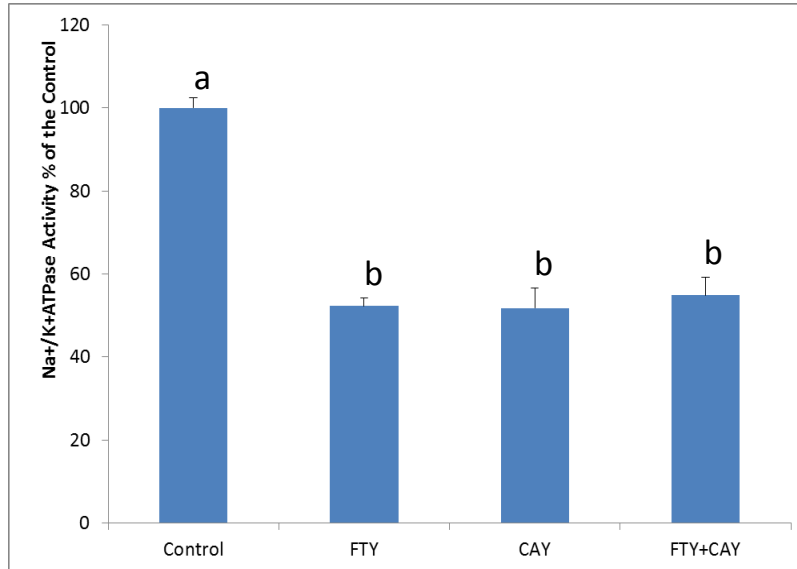


Figure 4a: FTY720P inhibits the activity of the Na⁺/K⁺ ATPase in HepG2 cells in presence of CAY10444. Values are means ± SEM. N=3. Bars not sharing the same letter are considered significantly different from each other at p<0.01 according to the Tukey-Kramer multiple comparison test.

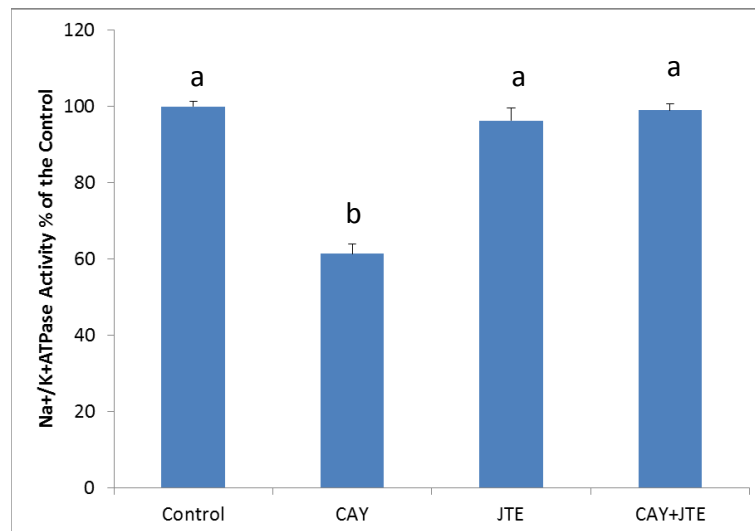


Figure 4b: JTE-013 blocks the effect of CAY10444 on the activity of the Na⁺/K⁺ ATPase in HepG2 cells. Values are means ± SEM. N=3. Bars not sharing the same letter are considered significantly different from one another at p<0.01 according to the Tukey-Kramer multiple comparison test.

C. Involvement of PKA

The effect of FTY720P on the activity of the Na⁺/K⁺ ATPase was studied in cells pre-treated with RpcAMP (Figure 6), a PKA inhibitor. The inhibitory effect of FTY720P on the Na⁺/K⁺ ATPase disappeared completely in the presence of this inhibitor and was mimicked by dbcAMP, a cell permeable cAMP analogue (Figure 5).

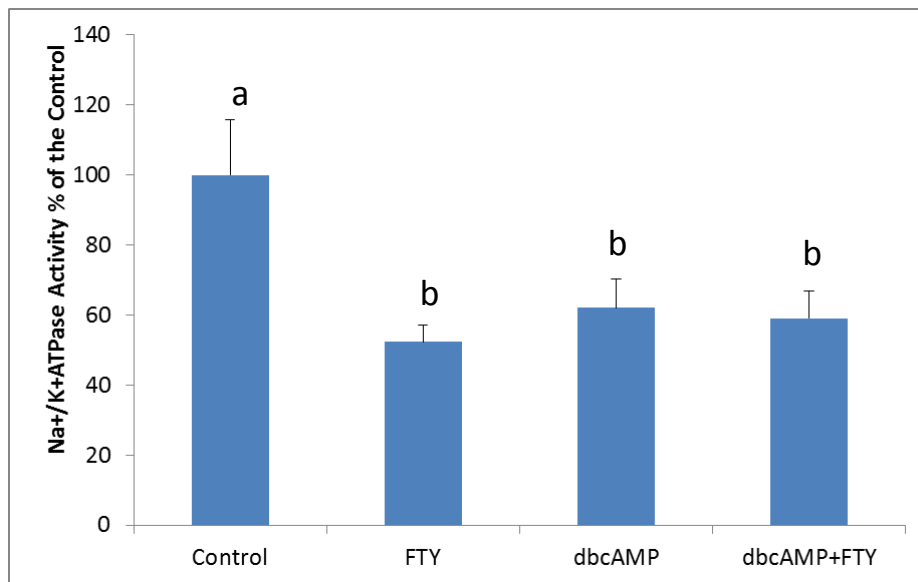


Figure 5: dbcAMP inhibits the activity of the Na⁺/K⁺ ATPase in HepG2 cells similar to FTY720P. Values are means \pm SEM. N=3. Bars not sharing the same letter are considered significantly different from one another at $p < 0.05$ according to the Tukey-Kramer multiple comparison test.

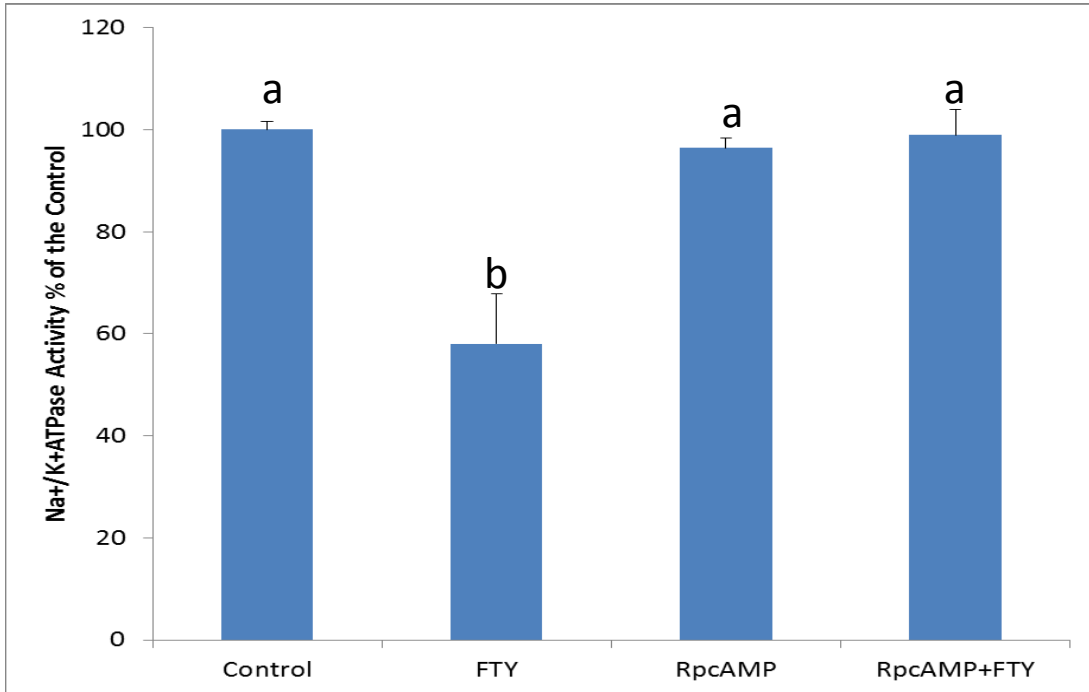


Figure 6: RpcAMP completely blocks the effect of FTY720P on the activity of the Na⁺/K⁺ ATPase in HepG2 cells. Values are means \pm SEM. N=3. Bars not sharing the same letter are considered significantly different from one another at $p < 0.05$ according to the Tukey-Kramer multiple comparison test.

D. Involvement of PKC

The inhibitory effect of FTY720P on the Na⁺/K⁺ ATPase disappeared completely in presence of calphostin, a PKC inhibitor (Figure 7). Treating the cells with phorbol 12-myristate 13-acetate (PMA) led to a similar inhibitory effect to that of FTY720P (Figure 8).

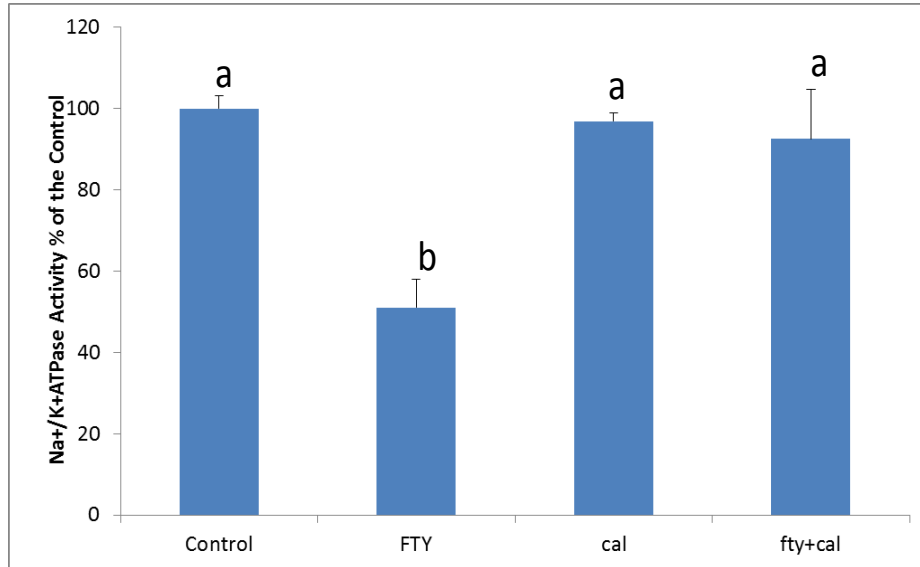


Figure 7: Disappearance of the effect of FTY720P on the activity of the Na⁺/K⁺ ATPase in HepG2 cells in presence of calphostin. Values are means ± SEM. N=3. Bars not sharing a common letter are considered significantly different from one another at p<0.05 according to the Tukey-Kramer multiple comparison test.

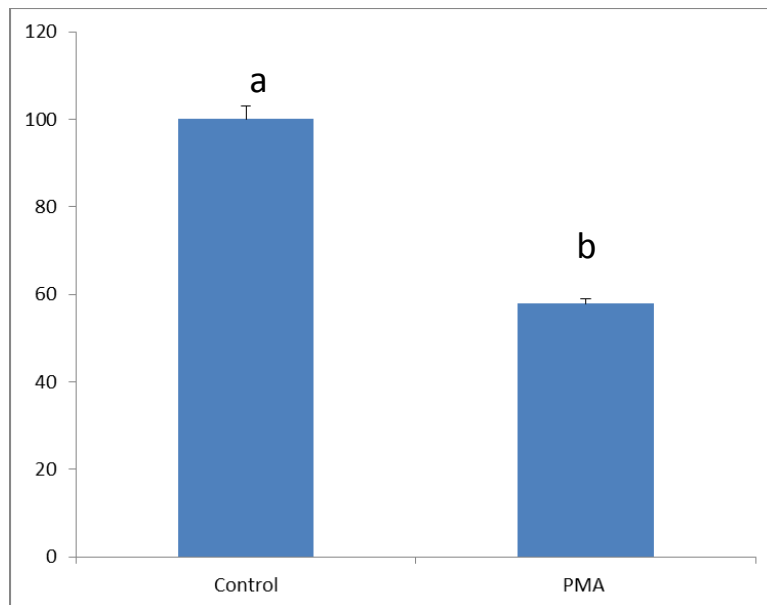


Figure 8: PMA inhibits the activity of the Na⁺/K⁺ ATPase in HepG2 cells. Values are means ± SEM. N=3. Bars not sharing the same letter are considered significantly different from each other at p<0.05 according to the Tukey-Kramer multiple comparison test.

E. Involvement of PGE₂ and the EP2 receptor

The inhibitory effect of FTY720P on the Na⁺/K⁺ ATPase disappeared completely in the presence of indomethacin a COX inhibitor (Figure 9). A similar inhibitory effect was observed in cells treated with exogenous PGE₂ (Figure 10).

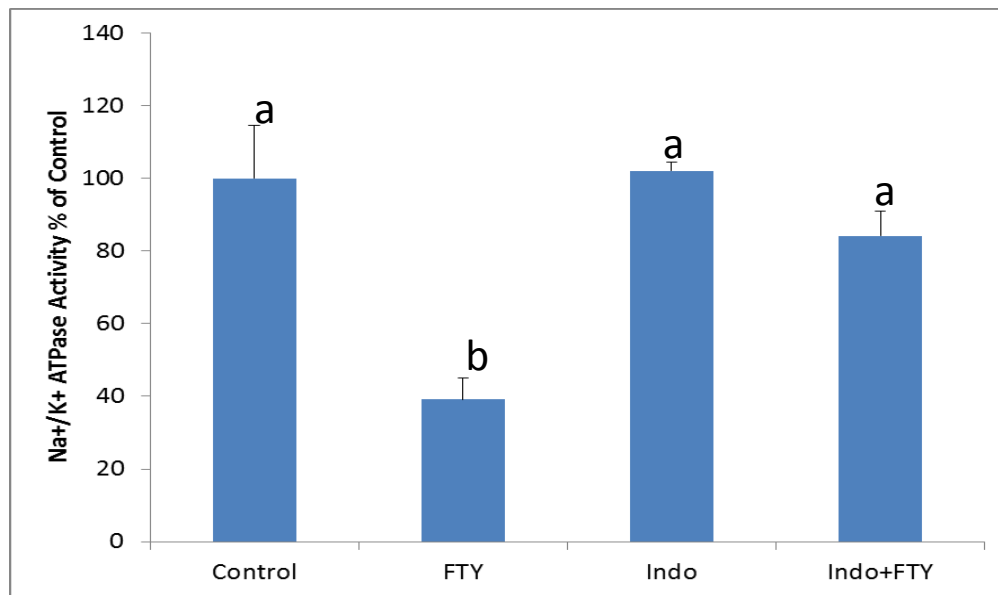


Figure 9: Indomethacin completely abolishes the effect of FTY720P on the activity of the Na⁺/K⁺ ATPase in HepG2 cells. Values are means \pm SEM. N=3. Bars not sharing the same letter are considered significantly different from each other at $p < 0.01$ according to the Tukey-Kramer multiple comparison test.

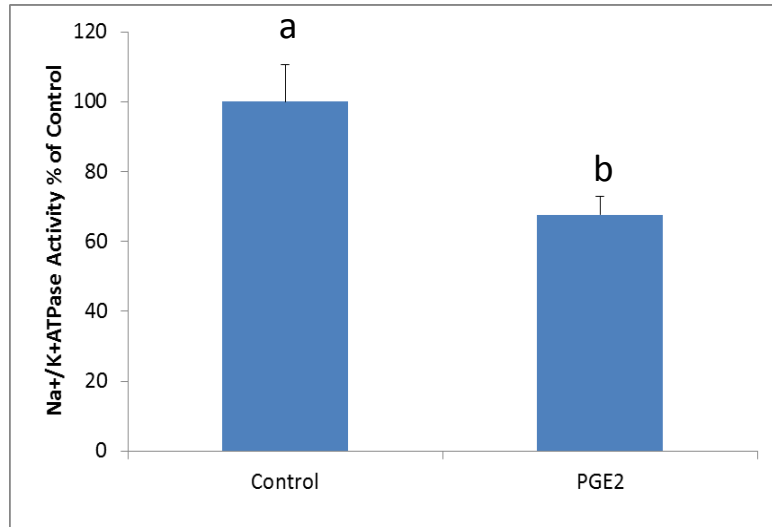


Figure 10: Exogenous PGE₂ inhibits the activity of the Na⁺/K⁺ ATPase in HepG2 cells. Values are means ± SEM. N=3. Bars not sharing the same letter are considered significantly different from each other at p<0.05 according to the Tukey-Kramer multiple comparison test.

HepG2 cells were treated with Butaprost, the EP2 receptor agonist in an attempt to identify the EP receptor involved in this signaling pathway. Butaprost mimicked the inhibitory effect of FTY720P suggesting the involvement of the EP2 receptor (Figure 11).

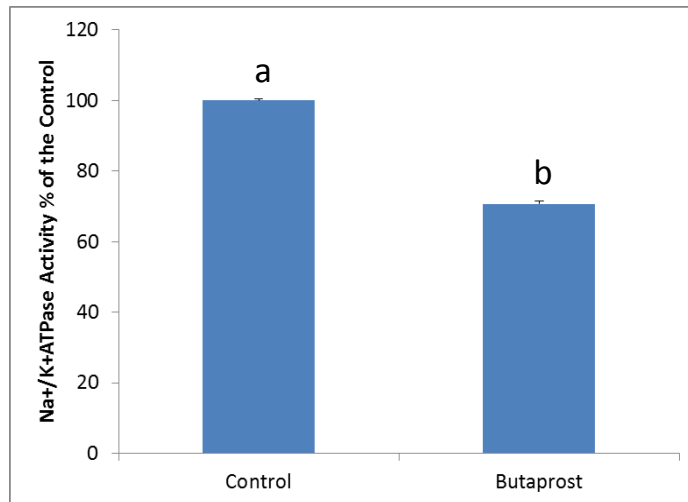


Figure 11: Butaprost mimics the inhibition of FTY720P on the activity of the Na⁺/K⁺ ATPase in HepG2 cells. Values are means ± SEM. N=3. Bars not sharing the same letter are considered significantly different from one another at p<0.01 according to the Tukey-Kramer multiple comparison test.

F. Locating PKC and PKA with respect to each other and to PGE₂

In order to locate PGE₂ with respect to PKC, cells were treated with PMA in presence of indomethacin. PMA on its own exerted a similar inhibitory effect on the Na⁺/K⁺ ATPase to that of FTY720P. This effect was, however, completely abolished in presence of indomethacin indicating that PKC is upstream of PGE₂ (Figure 12).

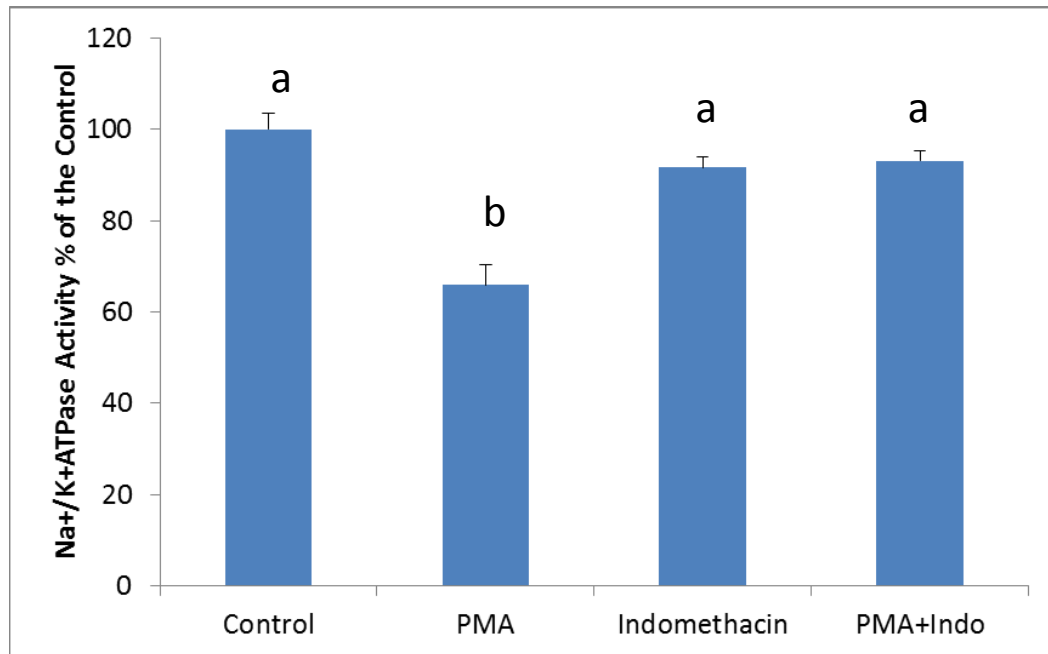


Figure 12: PKC is upstream of PGE₂. Values are means ± SEM. N=3. Bars not sharing the same letter are considered significantly different from one another at p<0.01 according to the Tukey-Kramer multiple comparison test.

In order to locate PGE₂ with respect to PKA, cells were treated with PGE₂ in presence of RpcAMP. The inhibitory effect of PGE₂ was completely abolished in presence of RpcAMP indicating that PKA is downstream of PGE₂. (Figure 13)

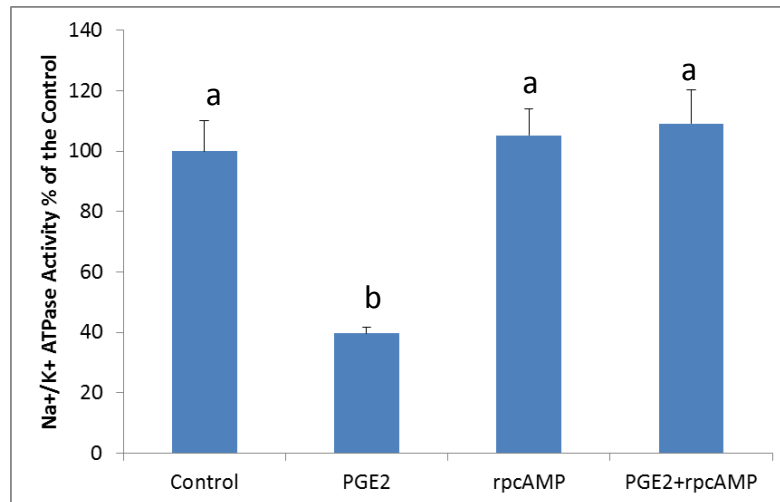


Figure 13: PGE₂ is upstream of PKA. Values are means ± SEM. N=3. Bars not sharing the same letter are considered significantly different from one another at p<0.01 according to the Tukey-Kramer multiple comparison test.

G. Involvement and location of NF-κB

The inhibitory effect of FTY720P did not appear in presence of NF-κB inhibitor. (Figure 14)

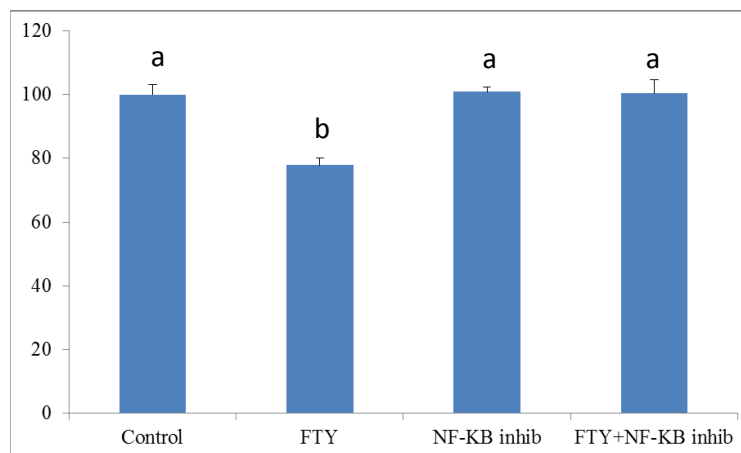


Figure 14: The effect of FTY720P on the activity of the Na⁺/K⁺ ATPase in HepG2 cells disappears in presence of NF-κB inhibitor. Values are means ± SEM. N=3. Bars not sharing the same letter are considered significantly different from each other at p<0.01 according to the Tukey-Kramer multiple comparison test.

Moreover, probing for I κ B using western blot analysis revealed a decrease in the levels of I κ B following FTY720P treatment. This further validates the involvement of NF- κ B in this signaling pathway. (Figure 15)

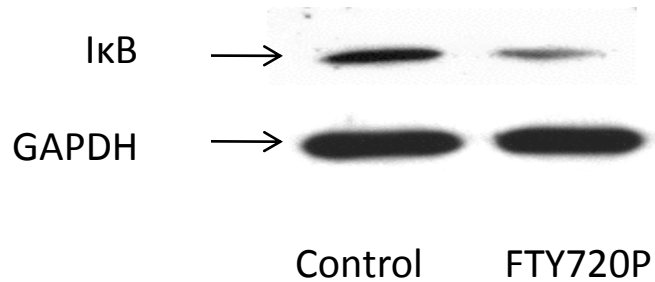


Figure 15: Western blot analysis showing the down-regulation of I κ B by FTY720P. (protein load: 40 μ g).

The inhibitory effect of PMA was no longer manifested in the presence of the NF- κ B inhibitor (Figure 17), but that of PGE₂ was unaltered by this inhibitor (Figure 16).

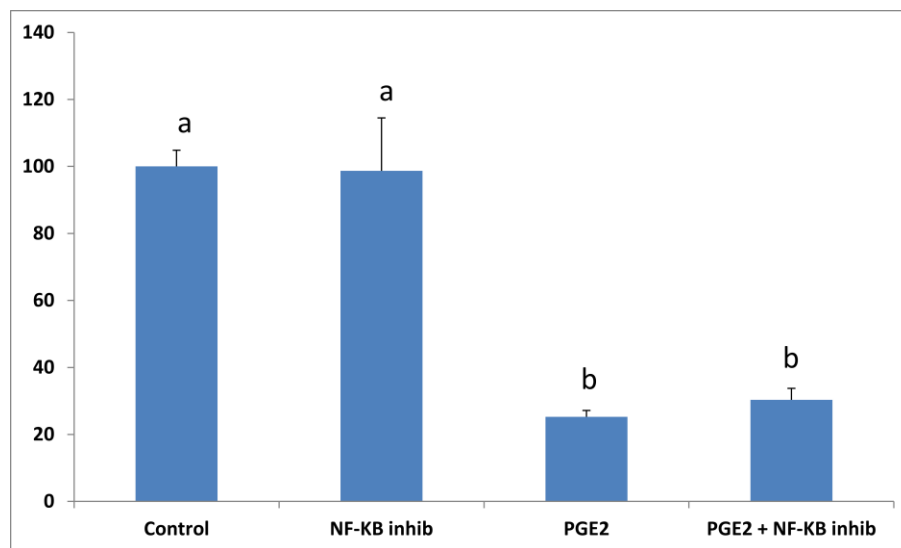


Figure 16: PGE₂ is downstream of NF- κ B. Values are means \pm SEM. N=3. Bars not sharing a common letter are considered significantly different from each other at $p < 0.01$ according to the Tukey-Kramer multiple comparison test.

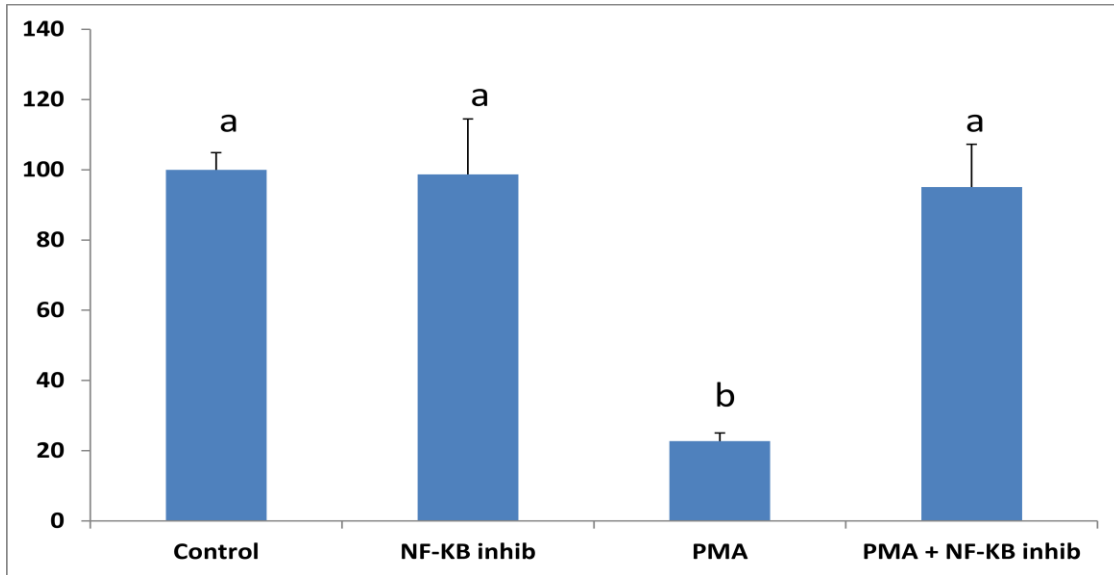


Figure 17: PMA is upstream of NF- κ B. Values are means \pm SEM. N=3. Bars not sharing a common letter are considered significantly different from one another at $p < 0.01$ according to the Tukey-Kramer multiple comparison test.

H. Involvement of Nitric oxide

The inhibitory effect of FTY720P on the Na^+/K^+ ATPase disappeared completely in the presence of PTIO, a NOS inhibitor, (Figure 18) but was mimicked by SNAP1, an NO generator. (Figure 9)

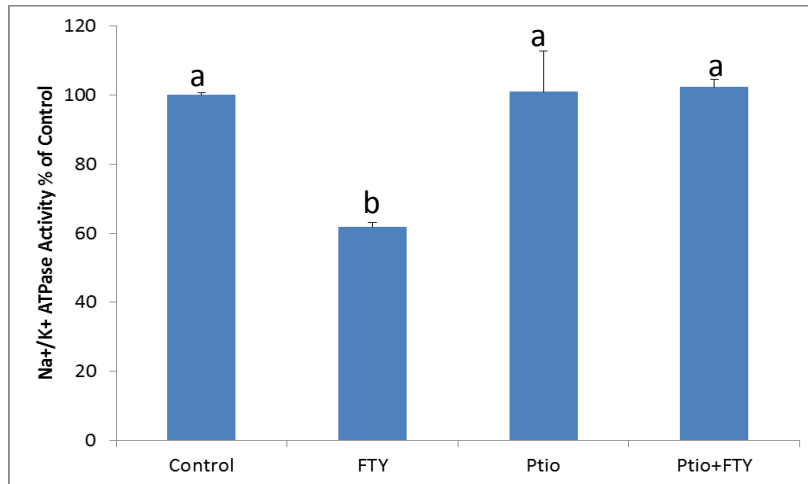


Figure 18: Effect of FTY720P on the activity of the Na⁺/K⁺ ATPase in HepG2 cells disappears completely in presence of PTIO. Values are means \pm SEM. N=3. Bars not sharing the same letter are considered significantly different from one another at $p < 0.05$ according to the Tukey-Kramer multiple comparison test.

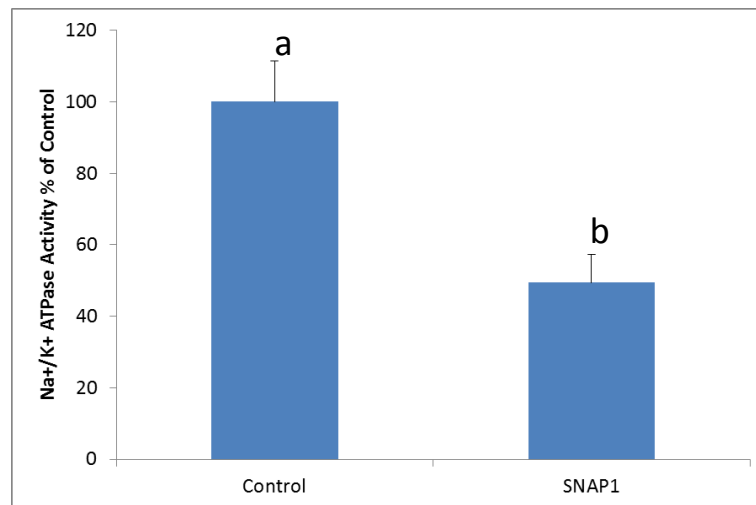


Figure 19: SNAP1 mimics FTY720P's action on the activity of the Na⁺/K⁺ ATPase in HepG2 cells. Values are means \pm SEM. N=3. Bars not sharing the same letter are considered significantly different from one another at $p < 0.05$ according to the Tukey-Kramer multiple comparison test.

I. Involvement of ERK1/2

In presence of PD98059a MEK1/2 inhibitor, the inhibitory effect of FTY720P on the Na^+/K^+ ATPase did not appear (Figure 20).

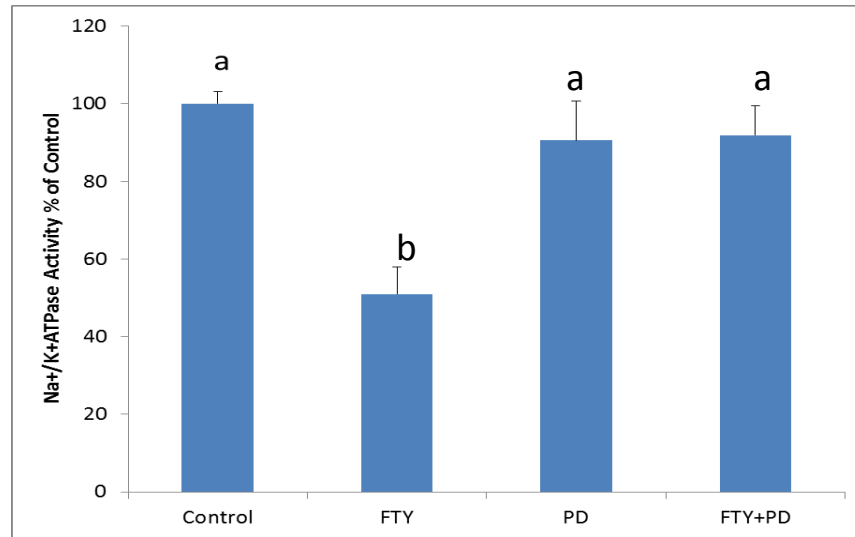


Figure 20: Effect of FTY720P on the activity of the Na^+/K^+ ATPase in HepG2 cells is blocked in presence of PD98059. Values are means \pm SEM. N=3. Bars not sharing the same superscript are considered significantly different from each other at $p < 0.05$ according to the Tukey-Kramer multiple comparison test.

J. Locating NO and ERK1/2 with respect to PGE_2

In the presence of PTIO, or PD98059 the effect of PGE_2 on the ATPase was completely abolished (Figure 21 and 22). Moreover, western blot analysis showed a higher protein expression level of p-ERK1/2 in PGE_2 treated cells (Figure 23).

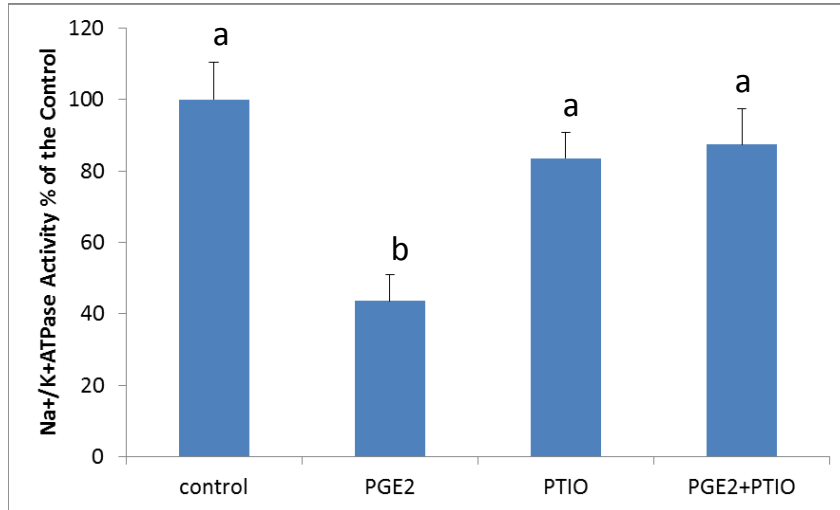


Figure 21: PGE₂ is upstream of NO. Values are means \pm SEM. N=3. Bars not sharing the same superscript are considered significantly different from each other at $p < 0.01$ according to the Tukey-Kramer multiple comparison test.

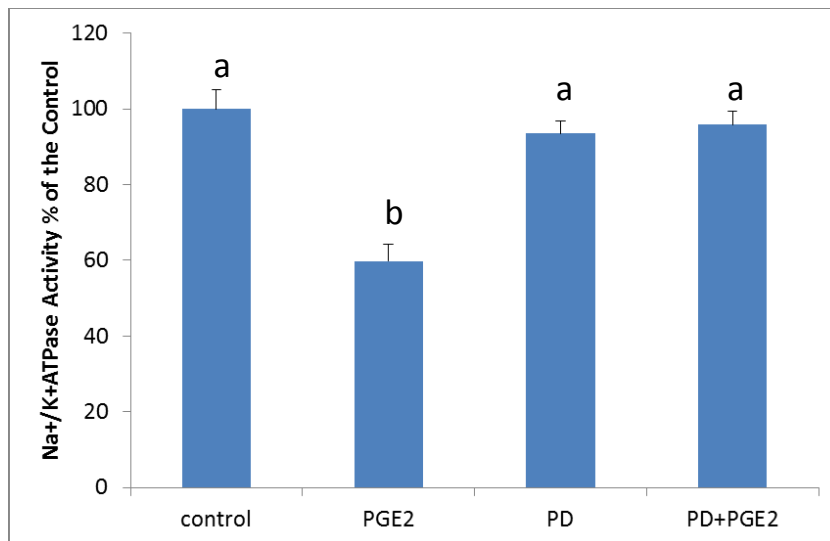


Figure 22: PGE₂ is upstream of ERK1/2. Values are means \pm SEM. N=3. Bars not sharing the same superscript are considered significantly different from one another at $p < 0.01$ according to the Tukey-Kramer multiple comparison test.

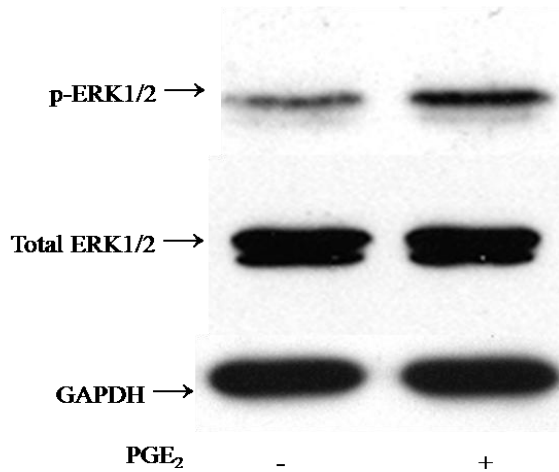


Figure 23: PGE₂ is shown through western blot analysis to be upstream of ERK1/2. (protein load:40μg).

K. Locating PKA, NO, and ERK1/2 with respect to each other

An increase in the levels of phospho-ERK1/2 but not total ERK was observed in cells treated with dbcAMP. However, this dbcAMP induced increase was completely abolished in presence of PTIO (Figure 24).

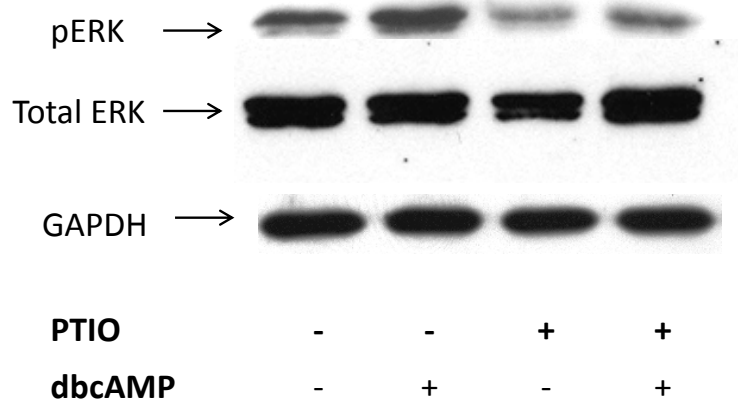


Figure 24: NO is shown through western blot analysis to be downstream of PKA and upstream of PGE₂. (protein load: 40μg).

L. Involvement of intracellular calcium

In order to determine whether calcium is involved in this signaling pathway, the effect of FTY720P was studied in cells treated with the calcium chelator, BAPTA-AM. The chelator did not alter the inhibitory effect of FTY720P (Figure 25).

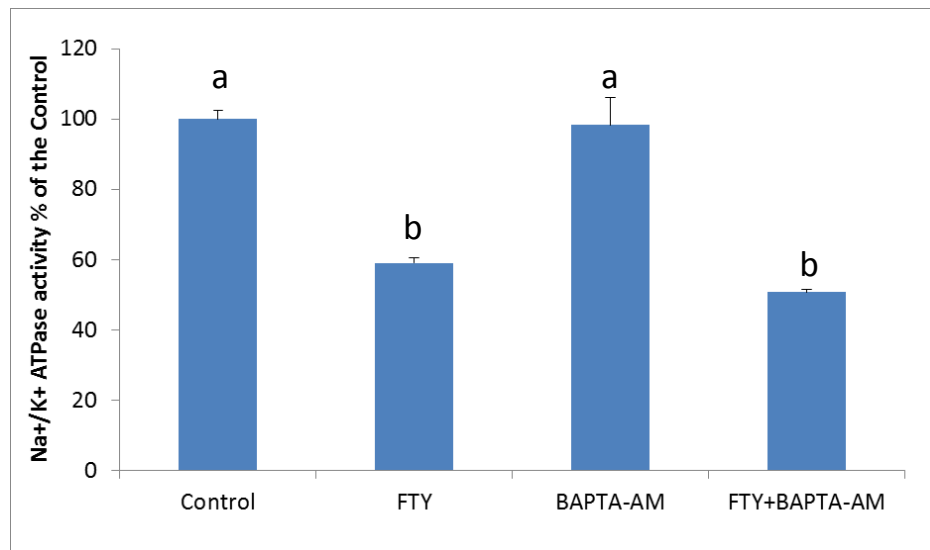


Figure 25: The studied signaling pathway is calcium independent. Values are means \pm SEM. N=3. Bars not sharing the same superscript are considered significantly different from one another at $p < 0.01$ according to the Tukey-Kramer multiple comparison test.

CHAPTER V

DISCUSSION

The Na⁺/K⁺ ATPase is implicated in bile synthesis, and any change in its activity is expected to alter bile secretion. On the other hand, S1P was shown previously to exert a significant inhibitory effect on hepatic Na⁺/K⁺ ATPase (Dakroub & Kreydiyyeh, 2012). The literature reports also a strong correlation between a reduced activity of the ATPase and apoptosis. Thus, through its effect on the ATPase, S1P may induce liver cell death and alter bile flow. However, the receptors and mediators involved in this inhibitory effect have not yet been identified. Knowing the mechanism of action of S1P would allow us to target the signaling intermediates, change their activity, and consequently alleviate any undesirable effect. This work is an attempt to delineate the signaling pathway using the S1P agonist, FTY720P.

A. Dose response study

A dose response study was conducted to determine the optimal dose at which FTY720P mimics the effect of S1P and inhibits the Na⁺/K⁺ ATPase (Figure 1). The most effective doses ranged between 7.5nM (48% inhibition) and 15nM (42% inhibition). No significant changes were observed at higher or lower doses probably because at low doses none of the receptors are activated, while at higher doses more than one type of receptor is activated. Since different receptors may act through opposite signaling cascades, their effects may cancel each other leading to no change in the activity of the Na⁺/K⁺ ATPase. It is important to note that FTY720P was used in this study instead of FTY720 because this molecule is only biologically active after

its phosphorylation by sphingosine kinases (Zemann, et al., 2006) in the liver. Therefore, we opted to directly administer the biologically active molecule to the cells.

B. FTY720P inhibits the Na⁺/K⁺ ATPase by activating the S1P2 receptor

The presence of the 5 different types of S1P receptors in HepG2 cells was confirmed by western blot analysis (Figure 2). Since the inhibitory effect of FTY720P was completely abolished by the S1P2 blocker, JTE-013, (Figure 3), it was concluded that the S1P2 receptor is the sole receptor activated by FTY720P in this signaling pathway. Activation of S1P2 was found to be behind the anti-proliferative role of S1P in hepatocytes. In fact, S1P2 null mice were shown to exhibit a higher rate of liver regeneration in comparison with wild type ones and the inhibitory effect of S1P on the proliferation of rat hepatocytes was abolished by the S1P2 blocker, JTE-013 (Ikeda, et al. 2003; Ikeda, et al. 2009). Activation of S1P2 was also found to counteract the proliferative effect of the platelet derived growth factor (PDGF) in embryonic fibroblasts (Goparaju, et al., 2005). This anti-proliferative effect of the S1P2 receptor is in accordance with our obtained results since an inhibition in the activity of the Na⁺/K⁺ ATPase has always been linked to anti-proliferative effects (Kaplan, 1978).

Although the effect of FTY720P was not altered by the S1P3 blocker, CAY10444, the blocker alone exerted an inhibitory effect on the ATPase (Figure 4a) that was not manifested in presence of the S1P2 blocker, JTE-013, (Figure4b) indicating that CAY10444 acts on the pump by activating the S1P2 receptor. This observation points to a role of CAY10444 as a S1P2 agonist at the concentration used in this study (17.4μM). Instances of ligands acting as a blocker for one S1P receptor and an agonist for another have been previously reported in the literature. For example, the S1P2 blocker, JTE-013, was shown to augment the excitability of sensory neurons

by activating the S1P1 receptor (Li, et al. 2012). CAY10444 seems to play an analogous role: it acts as an antagonist for S1P3 and an agonist for S1P2. Such a dual role for CAY10444 has not been reported before.

C. Involvement of PKA

The S1P2 receptor is known to be coupled to three different types of G-proteins namely, Gq, Gi, and G12,13 (Brinkmann, 2007). The Gi protein is known to down-regulate the adenylyl cyclase enzyme leading to a drop in intracellular cAMP levels (Hidlebrandt, et al. 1983). Dibutyl cAMP (dbcAMP) is a lipophilic cAMP analogue that readily penetrates the cell membrane (Rundfeldt, et al. 2012). Therefore, if FTY720P acts via S1P2 and Gi, then treatment with dbcAMP is expected to counteract the effects of Gi and lead to an opposite effect to that induced by FTY720P. However, as observed in Figure 5, the inhibitory effect of FTY720P was mimicked by dbcAMP and completely blocked by RpcAMP, a PKA inhibitor. The results indicate that activated S1P2 receptors do not act via Gi and that there is a cAMP dependent protein kinase (PKA) involved somewhere in the pathway. PKA may alter the activity of the ATPase by direct phosphorylation or indirectly by phosphorylating some intermediate signaling molecules. Such an involvement of PKA in the regulation of the Na⁺/K⁺ ATPase activity has been frequently reported. For instance, PKA was shown to mediate dopamine's inhibitory action on the Na⁺/K⁺ ATPase in cells of the cortical collecting duct via Gs (Satoh, et al.). This type of G protein is not known however, to be coupled to S1P2. The involvement of Gq was hence investigated.

D. Involvement of Gq:

Gq activates the phospholipase C enzyme which hydrolyzes PIP₂ into DAG and IP₃. DAG activates PKC directly while IP₃ activates it indirectly (Rhee, 2001). Therefore, we tested for the involvement of Gq in this signaling pathway by checking the involvement of PKC. The complete disappearance of the effect of FTY720P on the Na⁺/K⁺ ATPase in the presence of the PKC inhibitor, calphostin C, (Figure 7) and the imitation of this effect by the PKC activator, PMA, (Figure 8) provide sufficient evidence for the involvement of PKC and therefore Gq in the studied pathway. Moreover, the complete abolishment of the inhibitory effect of FTY720P indicates that the Gq protein is the only G protein activated by the S1P₂ receptor. Several examples of the inhibition of the Na⁺/K⁺ ATPase by PKC could be provided, one of which is dopamine's inhibitory action on the Na⁺/K⁺ ATPase in MDKC cells which was mimicked by PMA and abrogated by two different PKC inhibitors (Shahedi, et al. 1992).

E. Involvement of PGE₂ and the EP₂ receptor

PGE₂ was found in this work to be a mediator of the inhibitory effect of FTY720P on the Na⁺/K⁺ ATPase (Figures 9 and 10). Such an effect of the prostaglandin has been reported before. For example, the inhibition of the Na⁺/K⁺ ATPase by endothelin in cells of the inner medullary collecting duct was blocked by ibuprofen, a COX enzyme inhibitor, indicating a role of PGE₂ in this inhibition (Zeidel, et al. 1989). Moreover, indomethacin, another COX enzyme inhibitor, abolished the inhibitory effect of TNF-α on Na⁺/K⁺ ATPase in HepG2 cells while exogenous PGE₂ mimicked TNF-α's action by activating EP₂ receptors (Kreydiyyeh, et al. 2007). PGE₂ was also shown to participate in many of the S1P-induced signaling pathways. As a matter of fact, S1P was found to up-regulate COX-2 expression in rat and human mesangial cells by

activating its S1P2 receptor (Völzke, et al. 2014). S1P increased also COX-2 expression and PGE₂ production in L929 fibroblasts; these effects were abrogated by RNA interference (RNAi) against the sphingosine kinase 2 enzyme and enhanced by RNAi against the S1P lyase enzyme and the S1P phosphatase enzyme (Pettus, et al., 2003).

PGE₂ acts as an autocrine/paracrine agent by activating one of its four G-protein coupled EP receptors namely EP1, EP2, EP3, and EP4 (Narumiya, et al 1999). Since we already proved the involvement of PKA in the studied pathway, the two Gs coupled EP receptors, EP2 and EP4, were considered as potential candidate receptors. Our lab has demonstrated previously an EP2 mediated PGE₂ inhibitory effect on the Na⁺/K⁺ ATPase in HepG2 cells (Kreydiyyeh, et al. 2007). Consequently our choices were narrowed down to the EP2 receptor as the potential receptor involved in the FTY720P action. As expected, Butaprost, the EP2 receptor agonist, mimicked the inhibitory effect of FTY720P (Figure 11). However, a full confirmation of the involvement of the EP2 receptor awaits a treatment with an EP2 blocker prior to FTY720P treatment.

F. PGE₂ is downstream of PKC and upstream of PKA

Since the Gs-coupled EP2 receptor was suggested to be the one involved, PKA is expected to be one of its mediators. In fact, the inhibitory effect of exogenous PGE₂ completely disappeared in the presence of RpcAMP, a PKA inhibitor (Figure 13). Moreover, the S1P2 receptor was shown to act solely via Gq, consequently PKC is expected to be directly downstream of S1P2 receptor and upstream of PGE₂. This was verified by the complete abolishment of the inhibitory effect of PMA, the PKC activator, in the presence of the COX inhibitor, indomethacin (Figure 12). Many works reported a similar effect of PKC on COX enzymes in various cell types. COX-2 was

shown to be activated by PKC in RAW 264.7 cells (Giroux & Descoteaux, 2000), as well as in non-pigmented ciliary epithelial cells (Delamere, et al. 1997).

G. NF- κ B is downstream of PKC and upstream of PGE₂

NF- κ B is one of the transcription factors involved in the up-regulation of COX-2 in many cell types. In human synovial fibroblast cells, the TNF- α – induced increase in COX-2 expression and PGE₂ production disappeared in presence of the NF- κ B inhibitor, PDTC (Ke, et al., 2007). Similarly, in Swiss 3T3 fibroblasts, the Proinsulin C peptide up-regulation of the COX-2 enzyme was blocked by specific PKC and NF- κ B inhibitors indicating that the effect of the peptide is mediated through a PKC/NF- κ B dependent pathway (Kitazawa, et al. 2006). Our results suggest a similar involvement of NF- κ B (Figure 14 and 15) downstream of PKC (Figure 17) and upstream of PGE₂ (Figure 16) in the FTY720P signaling pathway. Whether NF- κ B increases directly the protein expression of COX-2 or indirectly by promoting the expression of an upstream intermediate cannot be determined from the current results available.

H. Involvement of Nitric oxide

Nitric oxide abrogated also the effect of FTY720P on the pump and proved also to be an intermediate signaling molecule (Figure 18 and 19). In support of our finding, the two NO donors, SNAP and NONOate inhibited in opossum kidney cells, the activity of the Na⁺/K⁺ ATPase in a dose and time dependent manner (Liang & Knox, 1999) while the carbachol induced inhibition of the Na⁺/K⁺ ATPase in bovine ciliary processes was mimicked by SNAP and abolished by the NOS inhibitor, L-NAME (Ellis, et al. 2001).

NO is also known to participate in S1P induced signaling pathways in various cell types. For instance, eNOS activation by TNF- α in human endothelial cells was blocked by inhibitors of sphingomyelinase, sphingosine kinase, the S1P1, and the S1P3 receptors (Mulders, et al. 2006).

I. ERK1/2 is involved in the effect of FTY720P on the pump

Our results also indicate that the inhibitory effect of FTY720P on the pump is mediated via ERK1/2 since it disappeared completely in presence of PD98059 (Figure 20) and are in line with already reported effect of ERK on the pump. In opossum kidney cells, the parathyroid hormone (PTH) was shown to inhibit the Na⁺/K⁺ ATPase through an activation of PKC that was mediated by ERK1/2. The inhibition of PKC did not alter the PTH induced activation of ERK1/2 but abolished the PTH induced inhibition of the Na⁺/K⁺ ATPase (Khundirmi, et al. 2005).

ERK1/2 was also reported to mediate S1P signaling pathways. The regulatory role of S1P on aldosterone secretion by the zona glomerulosa cells of the adrenal glands was shown to be mediated via a stimulation of ERK1/2 (Brizuela, et al., 2007).

J. NO and ERK1/2 are downstream of PGE₂

The inhibitory effect of PGE₂ on the Na⁺/K⁺ ATPase was completely abolished in the presence of the NOS inhibitor, PTIO, indicating that NO is downstream of PGE₂. PGE₂ has been reported previously to up-regulate the NOS enzyme through Gs coupled receptors. In microglial cells, COX inhibitors that blocked the production of endogenous prostanoids lead to a drop in iNOS expression (Minghetti, et al. 1997) while in breast cancer cells, the COX-2 enzyme was shown to up-regulate iNOS through the EP4 receptor. This up-regulation was mimicked by EP4 agonists and diminished by COX-2 inhibitors (Timoshenko, et al. 2004).

This work demonstrated also a PGE₂ induced up-regulation of Phospho-ERK1/2, which disappeared completely in the presence of PD98059, indicating that ERK1/2 is downstream of PGE₂ (Figure 22 and 23). This is in accordance with a number of reports demonstrating the activation of ERK1/2 by NO. For instance, levels of phospho-ERK1/2 were increased by 2.5 folds in Caco-2 cells following treatment with exogenous PGE₂, an effect that disappeared in the presence of an EP4 receptor blocker (Leone, et al., 2007).

K. NO is downstream of PKA and upstream of ERK

By probing for phospho-ERK1/2 through western blot analysis, it was shown that PKA activates ERK1/2 and hence is upstream of it, and that NO lies downstream of PKA and upstream of ERK1/2 since the dbcAMP-induced increase in pERK disappeared in presence of PTIO, a NOS inhibitor (Figure 24).

Thus, PKA is upstream of the NOS enzyme and activates it in this signaling pathway. The mechanism by which PKA activates NOS depends on the specific NOS isoform. The nNOS and eNOS isoforms can be directly phosphorylated by PKA through calcium-independent as well as calcium dependent pathways leading to an increase in their sensitivity to resting calcium/calmodulin levels (Adak, et al 2001; Butt, et al. 2000; Hurt, et al. 2012). PKA can also activate the iNOS isoform indirectly by a mechanism that involves the activation of NF- κ B. In fact, it has been shown that PGE₂ activates the iNOS enzyme via PKA which then activates NF- κ B. The latter in turn activates specific NF- κ B binding sites in the iNOS gene triggering its up-regulation (Chen, et al. 1999). Such instances of PKA/NF- κ B induced activation of iNOS have been reported in the literature (Shirakawa & Mizel, 1989; Muroi & Suzuki, 1993).

The activation of ERK1/2 by NO is not uncommon to the literature. For instance, the NO donor, DEA/NO, was shown to activate ERK1/2 in primary midbrain cells (Canals, 2003), and the NO donors, SNAP and FK409, also triggered the activation of ERK1/2 in cerebral Purkinje cells (Endo & Launey, 2003). Similarly, this work showed that NO is upstream of ERK1/2 and therefore activates it. This could be achieved, as was reported by Lander et al. (1993) through a nitric oxide activation of ras, a known upstream activator of ERK1/2 (Raman, et al. 2007). ERK 1/2 is therefore the most downstream mediator in the studied signaling pathway. The kinase can phosphorylate directly the alpha subunit of the ATPase leading to a decrease in its activity and/or endocytosis and subsequent degradation (Khundmiri, et al. 2004).

L. Involvement of intracellular calcium

BAPTAM-AM, the calcium chelator, did not alter the inhibitory effect of FTY720P on the Na^+/K^+ ATPase (Figure 25). This indicates that the studied signaling pathway is calcium-independent. Calcium is an intracellular mediator that is needed for the proper functioning of specific PKC and NOS isoforms. Therefore, our results suggest that the PKC isozyme involved in this pathway belongs to the group of novel PKCs which can be solely activated by DAG (Bollag, 2009). On the other hand, eNOS and nNOS, but not iNOS have been shown to require a rise in intracellular calcium for activation (Nathan & Xie, 1994). Several studies have suggested that the eNOS and nNOS can be activated by serine/threonine kinases such as PKA at resting calcium levels (Adak, et al 2001; Butt, et al. 2000; Hurt, et al. 2012). Therefore, eNOS or nNOS are probably the potential isoforms based on the observation that calcium is not involved in this signaling pathway and in light of several studies that have shown that ceramides can activate the eNOS enzyme in a calcium-independent manner (Igarashi, et al. 1999).

The signaling pathway through which FTY720P acts on the Na⁺/K⁺ ATPase is presented below.

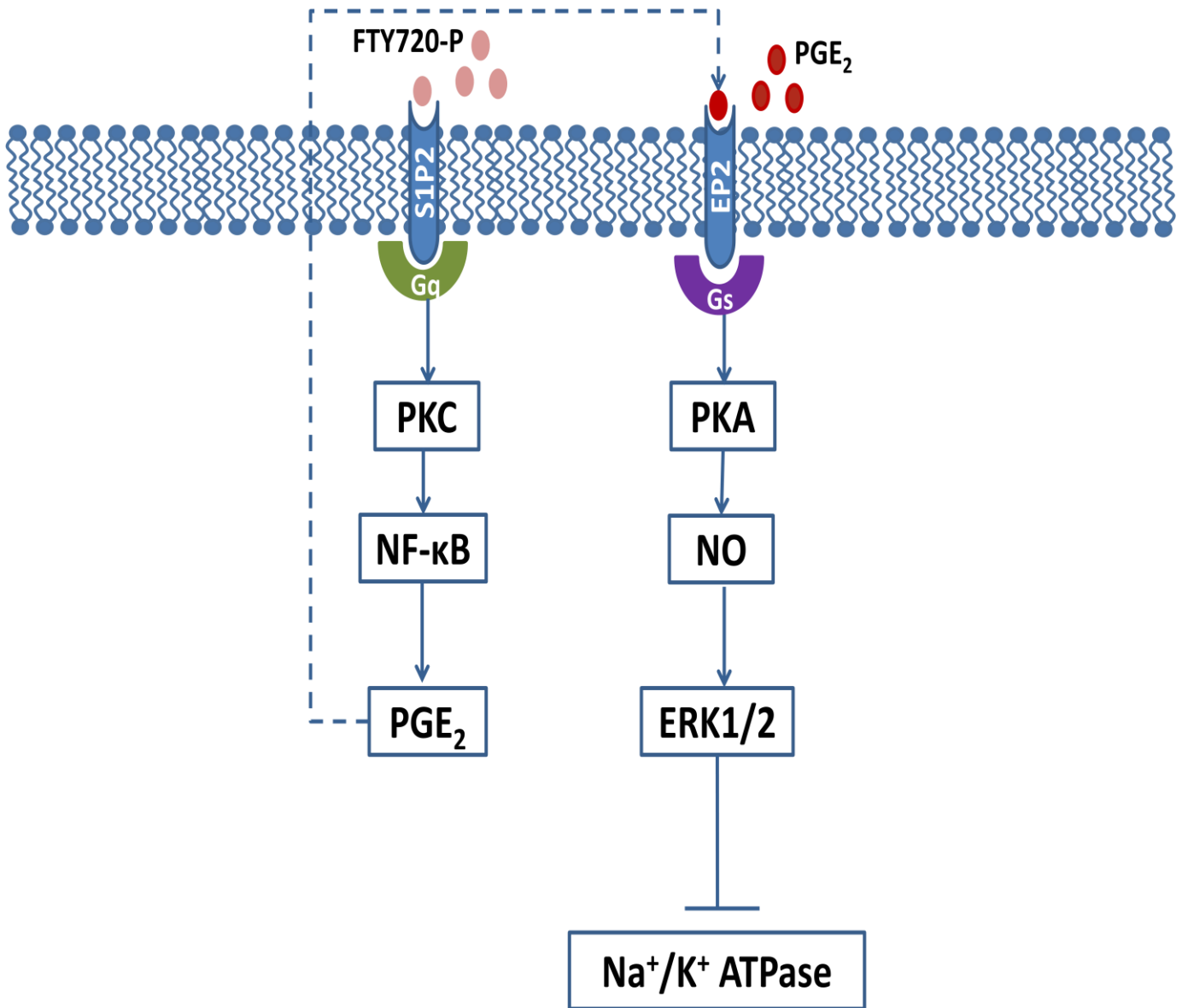


Figure 26 the signaling pathway of FTY720P on the hepatic Na⁺/K⁺ ATPase

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