AMERICAN UNIVERSITY OF BEIRUT

PROCYANIDIN B2 STIMULATES THE Na^+/K^+ ATPASE IN CACO-2 CELLS BY INDUCING PGE2 RELEASE

by
SUHEIR HASAN ZEINEDDINE

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by

SUHEIR HASAN ZEINEDDINE

Approved by:

Dr. Sawsan Kreydiyyeh, Professor — Advisor
Biology

Dr. Khouzama Knio, Professor — Member of Committee
Biology

Dr. Mike Osta, Associate Professor — Member of Committee
Biology

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AN ABSTRACT OF THE THESIS OF

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Procyanidins, are known to exert a wide spectrum of biological effects due to their anti-oxidant and anti-inflammatory properties. They are polyphenolic compounds present in plants and are highly abundant in grapes, apples, and cocoa. Procyanidins are polymers or oligomers of catechin or epicatechin, with varying degrees of polymerization. It was previously reported that procyanidins play a beneficial role in inflammatory bowel disease, a disease characterized by an abnormal immune response leading to inflammation of the intestine, and abnormal water absorption. Water transport in the colon follows ionic movements, mainly those of sodium whose gradient is established and maintained by the activity of the Na^+/K^+ ATPase or Na^+/K^+ pump. A decrease in the activity of the Na^+/K^+ ATPase was found to accompany inflammatory bowel disease and alter water transport across colonic cells. Thus, a correlation between procyanidins and the ATPase was suspected, and was examined in this work using Caco-2 cells as a model.

Cells were treated with Procyanidin B2 (17 μM, 2 hrs) and the activity of the ATPase was assayed by measuring the amount of inorganic phosphate liberated in presence and absence of ouabain, a specific inhibitor of the pump.

Procyanidin B2 activated the Na^+/K^+ ATPase but did not have any effect on its expression. Wortmannin, Calphostin C, and RpcAMP, respective inhibitors of PI3K, PKC, and PKA, abolished the stimulatory effect of Procyanidin B2, indicating that these molecules are along the signaling pathway. The effect of Procyanidin B2 did not appear also in presence of indomethacin, an inhibitor of COX enzymes, but a similar stimulatory effect was observed in presence of exogenous PGE2. This stimulatory effect was still manifested in the simultaneous presence of PGE2, and each of SC-19220, PF-04418948, and L-798106, respective blockers of EP1, EP2, and EP3 receptors, but abolished in presence of BGC-201531, an EP4 blocker. The results suggest that PGE2 acts through EP4.

Cells treated with PMA, a PKC activator, in presence of wortmannin, a PI3K inhibitor, still showed a significant increase in the activity of the Na^+/K^+ ATPase. The stimulatory effect of PMA was however abolished in presence of Indomethacin, indicating that PKC is downstream PI3K and upstream PGE2. PGE2 did not have any effect on the

ATPase in presence of RpcAMP, an inhibitor of PKA. SB 202190, a specific inhibitor of p38MAPK, increased the pump's activity, and an additive stimulatory effect was observed when cells were treated with a combination of PGE2 and SB 202190. The results infer that p38MAPK is not along the signaling pathway and that p38MAPK inhibits the pump at basal levels.

It was concluded that Procyanidin B2 activates PI3K leading to PKC activation, and PGE2 synthesis. The latter stimulates PKA through EP4 receptors, leading to the activation of the Na^+/K^+ ATPase.

Polyphenols and some procyanidins were reported to activate the bitter taste receptors (TAS2Rs) which are coupled to G-proteins and expressed in various tissues including the colon. We suspect that Procyanidin B2 acts on the pump through an interaction with TAS2Rs. This hypothesis would need however to be confirmed in the future.

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ABBREVIATIONS

AA	Arachidonic acid
AC	Adenylyl cyclase
AKAP	A-kinase anchoring protein
AKIP 1	A-kinase interacting protein 1
ALI	Acute lung injury
aPKCs	Atypical Protein Kinase Cs
ATCC	American type culture collection
ATP	Adenosine triphosphate
BH	Breakpoint-cluster-region homology
Btk	Bruton's tyrosine kinase
cAMP	Cyclic adenosine monophosphate
CD	Crohn's disease
COX	Cyclooxygenase
cPGES	Cytosolic prostaglandin E synthase
cPKCs	Conventional/Classical Protein Kinase Cs
CREB	cAMP responsive element binding protein
D/D domain	Dimerization and docking domain
DAG	Diacylglycerol
DMEM	Dulbecco's minimal essential medium
DMSO	Dimethyl sulfoxide
EC	Epicatechin
ECG	Epicatechin-3-gallate
EGF	Epidermal growth factor
EGR 1	Early growth response factor 1
EP	E-series prostaglandin receptor
ERK	Extracellular signal-regulated kinases
FBS	Fetal bovine serum
GAPDH	Glyceraldehyde-3-Phosphate Dehydrogenase
GDP	Guanosine diphosphate
GPCR	G-protein coupled receptor
Grb2	Growth factor receptor bound protein 2
GSE	Grape seed extract
GSK3β	Glycogen synthase kinase 3β
GTP	Guanosine triphosphate
HER2	Human epidermal growth factor receptor 2
TE	Hydroxyeicosatetraenoic acid
HRP	Horse radish peroxidase
Hsp70	Heat shock protein 70
IBD	Inflammatory bowel disease
IFL	Inter-flavan linkage
IGF-I	Insulin-like growth factor I

IL-1	Interleukin 1
IL-17	Interleukin 17
ILK	Integrin-linked kinase
INF-γ	Interferon γ
iNOS	Inducible nitric oxide synthase
i-SH domain	Inter SH domain
JNK	Jun N-terminal kinase
kDa	Kilodalton
LBPCs	Larch bark procyanidins
LPS	Lipopolysaccharides
LTB4	Leukotriene B4
MAPK	Mitogen activated protein kinase
MAPKAPK	MAPK activated protein kinase
mGLUR5	Metabotropic glutamate receptor 5
MIC	Minimal inhibitory concentration
MKKKs	MAPK kinase kinases
MKKs	MAPK kinases
mPGES	Microsomal/membrane bound prostaglandin E synthase
MRP4	Multidrug-resistance associated protein 4
MSK	Mitogen and stress activated protein kinase
mTOR	Mammalian target of rapamycin
MW	Molecular weight
NF-κB	Nuclear factor kappa B
NKA	Na^+/K^+ ATPase
NLS	Nuclear localization signal
NO	Nitric oxide
NOXO1	NADPH oxidase organizer 1
nPKCs	Novel Protein Kinase Cs
NSCLC	Non-small cell lung cancer
p38 MAPK	p38 mitogen activated protein kinase
PAR 6	Protease activated receptor 6
PAs	Proanthocyanidins
PB1	Phox and Bem 1
PBS	Phosphate buffered saline
PDE	Phosphodiesterase
PDK1	Phosphoinositide-dependent kinase 1
PG	Prostaglandin
PGT	Prostaglandin transporter
PH domain	Pleckstrin homology domain
PI3K	Phosphoinositide-3-Kinase
PKA	Protein Kinase A
PKB	Protein kinase B
PKC	Protein Kinase C
PKI	Protein kinase A inhibitor peptide

PLA	Phospholipase A
PLC	Phospholipase C
PLM	Phospholemman
PMA	Phorbol-12-myrsitate-13-acetate
PP2A	Protein phosphatase 2A
PPAR-γ	Peroxisome proliferator activated receptor γ
PS	Pseudosubstrate
PSC	Peanut seed coats
PtdIns	Phosphatidylinositols
RACC	Receptor-activated calcium channels
RhoGAP	Rho GTPase activating protein
RpcAMP	Adenosine-3′,5′-cyclic Monophosphorothioate Rp-Isomer Triethylammonium salt
SDS	Sodium dodecyl sulfate
SH domain	Src homology domain
Syk	Spleen Associated Tyrosine Kinase
TAB1	TAK 1 binding protein 1
TAK1	Transforming growth factor β activated protein kinase 1
TAS2Rs	Human Bitter Taste Receptors
TCF	T cell factor
TCR	T cell antigen receptor
TGF-β	Transforming growth factor β
TNF-α	Tumor necrosis factor α
TRP5	Transient receptor potential 5
TXA2	Thromboxane A2
UC	Ulcerative colitis
UPS	Ubiquitin proteasome system
VEGF	Vascular endothelial growth factor
Vps	Vacuolar protein sorting
VSMCs	Vascular smooth muscle cells
ZAP70	Zeta chain of T cell receptor associated protein kinase 70

CHAPTER I

INTRODUCTION

Proanthocyanidins (PAs) are the second most abundant group of natural phenolic compounds after lignin. They exist as oligomers or polymers of flavan-3-ol units (Gu et al., 2003; Santos-Buelga and Scalbert, 2000), and possess diverse biochemical properties, allowing them to interact with proteins, to chelate metals, and to act as antioxidants (Kennedy and Powell, 1985; Sarni-Manchado et al., 1999; Weber et al., 2007). PAs protect plants against predation and pathogens. Lately they were found to have significant beneficial effects to humans through their immunomodulatory, cardio-protective and anti-inflammatory properties (Sato et al., 1999; Subarnas and Wagner, 2000; Zhao et al., 2007). High MW polymeric cocoa procyanidins play an important role in the prevention of inflammatory bowel disease (IBD) and the reduction of its severity by reducing colonic inflammation and the loss of barrier function, which are considered main contributors to the pathogenesis of the disease (Bitzer et al., 2015a). One of the most prevalent symptoms of IBD is diarrhea, which is usually caused by a decrease in Na^+ absorption or an increase in Cl^- secretion, resulting in alterations in colonic water movements (Greig and Sandle, 2000; Sandle, 1998). The transport of several ions, basically Na^+, Cl^-, K^+, and HCO^-_3, is controlled by the Na^+/K^+ ATPase (NKA) which is an integral protein ubiquitously expressed in mammalian cells and localized on the basolateral membrane of colonocytes. It is responsible for the establishment and maintenance of the Na^+ and K^+ electrochemical gradient across the cell membrane and drives water movements by osmosis (Saha et al., 2015; Surawicz, 2010). It has been noted

that the NKA activity of intestinal cells is lowered in IBD (Magalhães et al., 2016), which raises the possibility that the beneficial protective role of procyanidins against colon inflammation may be mediated through an effect on the Na^+/K^+ ATPase. This study aims to test this hypothesis and to determine the signaling pathway using Caco-2 cells as a model.

CHAPTER II

LITERATURE REVIEW

A. Procyanidins/Proanthocyanidins

Proanthocyanidins (PAs), known also as condensed tannins, are present abundantly in some foods like cocoa, apples, and grapes (Hammerstone et al., 2000; Robbins et al., 2012) and had in the past some medical applications. They exist as oligomers or polymers of flavan-3-ol entities synthesized through the phenylpropanoid and the flavonoid pathways, and considered one of the most prevalent groups of plant phenolic compounds (De Bruyne et al., 1999; Dixon et al., 2005). PAs have a variety of biochemical and biological properties, such as protecting the plant from fungal and bacterial attacks, and predation by herbivores animals. They also have a role in limiting the growth of the nearby plants (Bais et al., 2003; Feeny, 1970; Koes et al., 2005; Scalbert, 1991). The stereochemistry and the hydroxylation of the chiral centers (thus the nature of flavan-3-ol units), the position and stereochemistry of the interflavan linkage (IFL) between monomers, and the degree of polymerization, are all factors that contribute to the variability in the structure of PAs (Dixon et al., 2005). Their degree of polymerization is relatively high and ranges from 7 to 190 in cider apple skin and pulp, and reaches up to 30 or more in brown or black soybean coat (Guyot et al., 2001; Takahata et al., 2001). The fate of the procyanidins in the human gastro-intestinal tract is still controversial. While some studies showed that dietary procyanidins are not depolymerized and released in the digestive system (Ottaviani et al., 2012a; Wiese et al., 2015), others showed their degradation and the absorption of the resulting monomers in the final part of

the intestine (Cooper et al., 2008; Fernández and Labra, 2013; Oleaga et al., 2013; Smith, 2013). A change of B-type procyanidins to epicatechin (Spencer et al., 2001b) was reported as well and the ability of a small percentage of dimeric procyanidins to cross the intestinal wall, and become 3'-O-methylated (Ottaviani et al., 2012b; Spencer et al., 2001a; Wiese et al., 2015).

Procyanidins were found to have various beneficial effects. Procyanidin B2 reduced in mice the undesirable pathological alterations that accompany diabetic nephropathy by downregulating the Akt signaling pathway (Yin et al., 2015; Zhang et al., 2013). It induced also the death of colorectal cancer cells through the PI3K/Akt/mTOR cascade which regulates apoptosis and autophagy (Zhang et al., 2019). In mice with LPS-induced acute lung injury (ALI), epicatechin (EC), the monomer of Procynaidin B1, was shown to exert a protective role by hindering the inflammatory injury via inhibition of the p38 MAPK-AP1 signaling pathway (Xing et al., 2019). Furthermore, procyanidins from grapes were found to induce lipolysis in 3T3-L1 adipocytes through activation of the peroxisome proliferator-activated receptor-γ (PPAR-γ) and protein kinase A (PKA) (Pinent et al., 2005), and grape seed procyanidin extract (GSE) was found to exert anticancer properties against non–small cell lung cancer (NSCLC), as a result of a reduced COX activity and PGE2 release (Sharma et al., 2010).

B. Na^+/K^+ ATPase

1. Structure and Tissue Distribution

The Na^+/K^+ pump or Na^+/K^+ ATPase (NKA) is the first member of the family of P-type ATPases (Skou, 1957). It is a transmembrane protein present in the basolateral

membrane of epithelial cells (Silva and Soares-da-Silva, 2012), that mediates the transport of 3 Na^+ ions outside the cell and 2 K^+ ions into the cell at the expense of the hydrolysis of one ATP molecule (Thomas, 1972), thus establishing and maintaining a sodium and potassium electrochemical gradient across the cell membrane.

NKA is made of an α-subunit and a β-subunit along with an additional γ-subunit expressed only in some tissues (Mercer, 1993).

The α-subunit (~110 kDa) has 3 cytoplasmic domains responsible for ATP hydrolysis named the N-domain (nucleotide binding), P-domain (phosphorylation), and A-domain (Actuator) along with 10 transmembrane segments responsible for ion transport across the membrane (Laursen et al., 2015; Laursen et al., 2013). Four isoforms of the α-subunit were identified (α_1-α_4): α_1 is ubiquitously expressed, α_2 is expressed in the skeletal muscles and in the heart, α_3 in the brain, and α_4 is restricted to the testis and present specifically in the spermatozoa (Kaplan, 1985; Mercer, 1993; Sweadner, 1989; Xie et al., 2013).

The β-subunit (~55 kDa) has one single pass segment, and is responsible for the proper assembly of the enzyme and its transport to the plasma membrane. Three isoforms exist for the β-subunit: β_1 which is ubiquitously expressed, β_2 is restricted to the heart and skeletal muscles, and β_3 which is found in the testes and the central nervous system (Kotyk and Amler, 1995).

The γ-subunit (~12 kDa) is a single pass hydrophobic segment present in specific tissues. It belongs to the family of FXYD proteins and it is thought to exert a regulatory function on the pump (Morth et al., 2011; Suhail, 2010; Therien and Blostein, 2000).

The isoforms of the α and β subunits can form various combinations resulting in 12 possible isozymes of NKA possessing diverse pharmacological and transport characteristics (Blanco and Mercer, 1998; Crambert et al., 2000).

NKA stabilization at the plasma membrane is maintained through ankyrin, a protein that interacts through its N-terminal end with the cytoplasmic domain of the α-subunit, and attaches the pump to the spectrin-based membrane skeleton (Jordan et al., 1995; Rubtsov and Lopina, 2000).

2. Regulation

The Na^+/K^+ ATPase plays many important physiological roles, and any defect in its function may cause severe disorders (Geering, 2006). This is why the activity of the Na^+/K^+ ATPase is regulated by several factors including cytokines, hormones and enzymes.

Pro-inflammatory cytokines such as IFN-γ, TNF-α, and IL-1β reduce the activity and expression of the NKA (Bertelsen et al., 2004; Kreydiyyeh and Al-Sadi, 2002b; Magro et al., 2005a; Markossian and Kreydiyyeh, 2005a; Sugi et al., 2001b). While the activity of colonic NKA is reduced by IL-1β (Kreydiyyeh and Al-Sadi, 2002a), TNF-α, and PGE2 (Markossian and Kreydiyyeh, 2005b; Musch et al., 2002), it was enhanced by TGF-β and NH_2Cl (Magro et al., 2005b; Schultheiss et al., 2005), and not affected by 5-Hydroxyeicosatetraenoic acid (HETE) and Leukotriene B4 (LTB4), the products of

lipoxygenase (Allgayer et al., 1988). Moreover, elevated levels of nitric oxide were shown to inhibit the NKA in T84 cells (Boughton-Smith et al., 1993; Sugi et al., 2001a). NKA was also downregulated in the epithelium of renal tubules upon exposure to LPS of gram-negative bacteria involved in IBD, due to high levels of reactive nitrogen species produced as a result of an increase in iNOS activity (Caradonna et al., 2000; Mézešová et al., 2013; Schmidt et al., 2007; Seven et al., 2005). Some studies also showed that in rabbit, chronically inflamed small intestine showed a lower expression of NKA in the basolateral membrane due to downregulation of ankyrin protein and depolarization (Saha et al., 2015).

The NKA activity is also modulated by various kinases. Phosphatidylinositol-3-kinase (PI3K) enhanced the activity of the NKA in vascular smooth muscle cells (VSMCs) treated with Insulin-like growth factor I (IGF-I) (Li et al., 1999), while the effect of Protein Kinase A (PKA) varied between inhibition and activation through a higher recruitment to the cell membrane (Bertorello et al., 1991; Carranza et al., 1998; Meister et al., 1989). PKC promoted an increase in the activity and expression of NKA in alveolar epithelial cells by inducing exocytosis of NKA units from late endosomes (Ridge et al., 2002). NKA was also downregulated by p38 MAPK and PGE2 (Al-Sadi and Kreydiyyeh, 2003). Increased calcium levels in cardiomyocytes enhanced strongly the cardiac NKA activity (Lu et al., 2016).

Phospholemman (PLM), a member of the FXYD family, co-localizes with the Na^+/K^+ ATPase, Na^+/Ca^{2+} exchanger and L-type Ca^{2+} channels, and is known to regulate ion transport in cardiac and skeletal muscle cells. Interaction of NKA with proteins of the FXYD family doesn't seem to have an effect on the pump expression, however it affects the transport activity of specific isoforms in specific tissues. The activity of the Na^+/K^+ ATPase is

modulated indirectly by modifications of PLM by kinases (Cheung et al., 2010; Geering, 2006). Indeed, phosphorylation of the phospholemman by cAMP-dependent protein kinases (PKAs) may relieve the inhibition exerted by the phospholemman on the pump (Bibert et al., 2008; Han et al., 2010; Mishra et al., 2015) and increase its affinity for cytoplasmic Na^+ (Han, 2006). PKA may exert also phosphlemman-independent effects on NKA. In cardiac myocytes, PKA modulation of NKA varied widely. While inhibition was noted by Galougahi et al., 2013, activation (Kockskämper et al., 2000) or no effect (Fine et al., 2013; Ishizuka and Berlin, 1993; Main et al., 1997) was reported by other studies. Some works related the effect of PKA to calcium levels and showed pump inhibition by cytosolic calcium and activation in its absence (Gao et al., 1996). Phosphorylation of the phospholemman by protein kinase C (PKC) in cardiac myocytes also leads to stimulation or inhibition (Gao et al., 1999; Han, 2006; White et al., 2009). It was suggested that its phosphorylation by PKCs induces the highest pump activity (Han, 2006) as compared to phosphorylation by other kinases. There are other regulatory mechanisms for NKA such as the palmitoylation of the phospholemman (Tulloch et al., 2011) and the redox-dependent glutathionylation of the β-subunit (Liu et al., 2012).

Additional modulation of the NKA can occur via certain lipids that specifically bind to the ATPase or to other proteins in the membrane and may interfere with the enzymatic activity, ligand binding, trafficking, and protein stability. The impact of the interaction of lipids with these proteins could be an assortment of several effects as well (Andersen and Koeppe, 2007; Contreras et al., 2011; Cornelius, 2011; Lee, 2011; Marsh, 2008). Actually, it was shown that cholesterol increases the hydrolytic activity of NKA, with the optimal activity

observed at 20 mol% cholesterol (Cornelius, 1995), and in its absence, ω-3 poly-unsaturated phospholipids caused an inhibition of NKA (Cornelius, 2008).

While some peptide hormones or neurotransmitters may alter the cell surface expression of NKA by inducing its phosphorylation by kinases (Therien and Blostein, 2000), steroid hormones like aldosterone increase the number of NKA units by altering gene transcription (Féraille and Doucet, 2001).

The ultimate consequence of NKA activity reduction is the buildup of intracellular Na^+ leading to an increase in cell volume, and the downregulation of Na/K/Cl cotransporter in intestinal cells and occludins expression, thus affecting the permeability of tight junctions and Cl^- secretion (Sugi et al., 2001a).

C. Protein Kinase C

Protein kinase C (PKC) is a member of the AGC family of lipid-sensitive serine/threonine protein kinases, involved in several cellular processes such as differentiation, proliferation, adhesion, migration, and apoptosis. Based on their mode of activation, the 10 members of the PKC family are divided into 3 groups: Conventional/classical PKCs (cPKCs), novel PKCs (nPKCs), and atypical PKCs (aPKCs). The conventional PKCs (α, βI, βII and γ) have a C1 domain (C1A and C1B) that serves as a binding site for diacylglycerol (DAG) and phorbol 12-myristate 13-acetate (PMA), and another C2 domain responsible for the Ca^{2+}-dependent membrane binding property (Griner and Kazanietz, 2007; Steinberg, 2008). The novel PKCs (δ, θ, ε, η) also have C1 and C2 domains, with the C2 domain lacking the residues implicated in the interaction with Ca^{2+}

compared to cPKCs. The atypical PKCs (ζ, λ/ι) have an atypical C1 domain which binds to phosphatidylinositol 3,4,5-trisphosphate (PIP3) or ceramide for activation, and a PB1 domain (Phox and Bem1) which is responsible for protein-protein interaction with other scaffold proteins that contain PB1 such as p62, MEK5 and PAR6 (Moscat et al., 2006a; Moscat et al., 2006b).

The PKC isozymes have a common structure comprising an N-terminal regulatory segment (~35 kDa) and a C-terminal kinase domain (~45 kDa) linked together by a hinge region. All PKC isozymes share an autoinhibitory pseudosubstrate fragment found in the regulatory segment. The second messenger-binding or protein-binding segments that are specific to each PKC subclass, determine the position of the autoinhibitory pseudosubstrate fragment which can switch between the inside and the outside of the substrate-binding cavity (Newton, 2018). The catalytic domain is auto-inhibited in the absence of cofactors by the regulatory domain, which contributes to this auto-inhibition, partly through the pseudosubstrate (PS) sequence motif (House and Kemp, 1987).

All PKCs need to be subjected to a set of phosphorylation processes after being synthesized in order to reach their auto-inhibited, stable form that is ready to respond to second messengers. It was shown that conventional PKCβII acquires an open conformation after its synthesis, with its membrane-targeting segments exposed, and its pseudosubstrate not bound to the kinase domain (Newton, 2018). The chaperone Hsp90 binds this open conformation and permits its phosphorylation (Gould et al., 2009). Next, the open PKC is phosphorylated at a conserved Thr residue, by the phosphoinositide-dependent kinase PDK-1 (Newton, 2010; Parker and Parkinson, 2001; Taylor and Kornev, 2011). This step

induces two phosphorylations at the C-terminal tail, and the fully phosphorylated form undergoes conformational changes where the C1 and C2 domains get masked, and the pseudosubstrate inserted in the substrate binding site (Newton, 2018). Novel PKCs are phosphorylated at their activation loop by PDK-1 (Cenni et al., 2002), with only PKC-ε requiring mTORC2 for the phosphorylation events (Facchinetti et al., 2008; Ikenoue et al., 2008). Atypical PKCs undergo co-translational phosphorylation at their turn motif by ribosome-associated mTORC2, where this step is followed by phosphorylation by PDK-1 at the activation loop; these phosphorylation steps happen in a similar fashion to those of Akt (Facchinetti et al., 2008). Some studies reported that PKCs may undergo other post-translational modifications including phosphorylation at Ser/Thr sites or at Tyr sites, in addition to ubiquitination and acetylation processes (Konishi et al., 1997).

Different signals activate specific subfamilies of PKC isozymes. Conventional PKCs are activated by Ca^{2+} and diacylglycerol (DAG) generated after the hydrolysis of PIP2. Ca^{2+} binds the C2 domain leading to the exposure of the binding site of PIP2: a plasma membrane lipid. The isozyme is thus recruited to the plasma membrane. Diacylglycerol inserted in the membrane as well, binds to the C1B domain, which induces a conformational change that leads to the expulsion of the pseudosubstrate from the substrate binding site (Newton and Johnson, 1998; Orr et al., 1992).

Diacylglycerol alone activates novel PKCs, where it can be translocated to different sites including the mitochondria, plasma membrane, Golgi, or the nucleus in the case of PKCδ. The high levels of DAG in the Golgi membranes favor the translocation of nPKCs to the Golgi, promoting continuous signaling by nPKCs (Gallegos et al., 2006).

Diacylglycerol and Ca^{2+} are not involved in the activation of atypical PKCs. Their activation is mediated by the interaction of their PB1 domain with the PB1 domains of protein scaffolds such as p62 and Par6; such interaction recruits aPKCs to their substrates and releases the pseudosubstrate from the substrate-binding pocket (Drummond and Prehoda, 2016).

PKCs are downregulated by several factors. Phorbol esters and bryostatins, acting as ligands for the C1 domain, stabilize PKC in an open conformation at the membrane, resulting in its dephosphorylation and degradation (HANSRA et al., 1999; Szallasi et al., 1994). Phosphorylated PKC-α is usually sumoylated at the Lys65 residue in the kinase domain, however dephosphorylation reduces sumoylation, promoting ubiquitination and degradation (Wang et al., 2016). It is noteworthy that the chaperone Hsp70 may rescue the dephophorylated enzyme by stabilizing it, through binding to the dephosphorylated turn motif, thus inducing its rephosphorylation and activity (Newton, 2018).

PKC isozymes have several downstream substrates, including membrane proteins. PKC was shown to phosphorylate a Thr654 on the EGF receptor, reducing its tyrosine kinase activity and ligand binding affinity, and inducing receptor internalization (Hunter et al., 1984; Livneh et al., 1988; Livneh et al., 1987; Santiskulvong and Rozengurt, 2007). Another substrate of PKC is the proto-oncogene HER2 which gets internalized upon phosphorylation (Ouyang et al., 1998). G-protein coupled receptors such as β-adrenergic receptors, as well as histamine, dopamine, and muscarinic receptors are also phosphorylated by PKCs resulting in their desensitization (Fujimoto et al., 1999; Hosey et al., 1995; Namkung and Sibley, 2004; Pitcher et al., 1992). PKCε was shown to reduce the

surface expression of metabotropic glutamate receptor mGluR5 (Schwendt and Olive, 2017), while PKC-α mediated the inhibitory phosphorylation of the catalytic subunit of phosphatidylinositol-3-kinase (PI3K) (Sipeki et al., 2006), and promoted the deactivation of Akt by inducing its dephosphorylating by PP2A (Tanaka et al., 2003).

D. Prostaglandin E2

1. Prostaglandin E2 properties and synthesis

Eicosanoids, comprising prostaglandins and leukotrienes, are active lipid molecules exerting a broad spectrum of biological effects in different pathological conditions including cancer and inflammation. PGE2 is a prostanoid, belonging to a subfamily of eicosanoids that comprises PGF2α, PGD2, PGI2 and thromboxane A2 (TXA2) as well. The synthesis of prostanoids starts with the action of phospholipases (PLAs) that mediate the hydrolysis of membrane phospholipids, thus releasing free fatty acids such as arachidonic acid (AA) (Dong et al., 2003; Dong et al., 2004; Ilsley et al., 2005; Legler et al., 2010; Wang et al., 2007). The liberated AA is then transformed by cyclooxygenase enzymes (COX) into prostaglandin H2 (PGH2), which is converted in turn to prostaglandin D2, prostaglandin E2, prostaglandin F2α, prostaglandin I2 or thromboxane A2 under the effect of their respective synthases PGDS, PGES, PGFS, PGIS, and TXAS (Menter et al., 2010; Wang and DuBois, 2010; Wang et al., 2007).

Three isoforms exist for COX enzymes (COX1-3): COX-1, which is expressed constitutively, COX-2, whose expression is induced by certain cytokines and growth factors and COX-3 which is a splice variant of COX-1 (Dannenberg and Subbaramaiah, 2003; Kundu et al., 2001; Legler et al., 2010). COX-2 is known to be implicated in the

regulation of inflammatory responses and its expression is enhanced greatly in the course of tumor progression, like in breast and colon cancers.

PGE synthases (PGES) are classified into two groups: cytosolic (cPGES) and microsomal/membrane bound (mPGES). cPGES are mainly associated with COX-1, and mPGES are favorably coupled to COX-2. mPGES has two isoforms mPGES-1 and mPGES-2: like COX-2, mPGES-1 expression is upregulated by pro-inflammatory cytokines and is considered the principal synthase that accounts for the elevated levels of PGE2 during inflammation and tumorigenesis (Menter et al., 2010; Nakanishi et al., 2010; Wang et al., 2007).

PGE2 is transported outside the cell after the completion of its synthesis via MRP4, a particular multidrug resistance-associated protein (MRP). Following its export, PGE2 binds specific cell surface E-series prostaglandin receptors (EP receptors), through which it mediates its biological functions, either in an autocrine or a paracrine manner. Next, PGE2 metabolism is carried out, starting by its transport to the cytoplasm passively, or actively via the prostaglandin transporter (PGT).

2. EP receptors

PGE2 receptors (EP receptors) belong to the family of G-protein coupled receptors (GPCR), consisting each of seven transmembrane domains, an extracellular N-terminus, an intracellular C-terminus, and three interhelical loops on each of the two sides of the membrane. G proteins associated with these receptors are activated upon binding of the first messenger and induce various intracellular signaling pathways (Oldham and Hamm,

2008). G proteins are heterotrimers formed of three distinct α, β and γ subunits that, in the inactive state, are present as a $G_{\beta\gamma}$ monomer and a guanine diphosphate-bound G_{α} subunit. After ligand binding, the receptor is activated and GDP is exchanged for GTP on the Gα subunit, thus lowering the affinity of Gα for Gβγ. This will lead to the dissociation of the heterotrimer, releasing free Gβγ and GTP-bound Gα subunits that interact with corresponding target molecules. G proteins are classified into four families according to the signaling activity and sequence identity of their subunits: Gαs, Gαi, Gαq/11 and Gα12/13 (Lappano and Maggiolini, 2011; Oldham and Hamm, 2008).

The E-series of prostaglandin receptors (EP receptors) has four subtypes named EP1, EP2, EP3 and EP4 to which PGE2 binds (Fulton et al., 2006; Menter et al., 2010; Nakanishi et al., 2010; Sugimoto and Narumiya, 2007; Wang et al., 2007). Each of these receptors has different tissue distribution, and induces specific intracellular signaling cascades (Sugimoto and Narumiya, 2007).

EP receptors have different affinities for PGE2: EP1 and EP2 are considered to be low affinity receptors in comparison to EP3 and EP4. High affinity receptors are probably activated at low PGE2 levels, however low affinity receptors are thought to be only activated in pathological situations when COX-2 expression and PGE2 synthesis are induced, such as in the early stages of skin tumor progression (Rundhaug et al., 2011).

a. <u>EP1 receptor</u>

EP1 receptor activation usually induces an increase in the level of intracellular calcium mediated via Gq and/or G11, as well as receptor-activated Ca^{2+} channels (RACC)

(Katoh et al., 1995; Tabata et al., 2002). Binding of PGE2 to Gq linked EP1 receptors activates phospholipase C (PLC) which hydrolyzes phosphatidylinositol-4,5-bisphosphate, into inositol 1,4,5-trisphosphate (IP3) that promotes Ca^{2+} mobilization, and diacyglycerol (DAG) that induces the activation of PKC (Fukami et al., 2010). The transient receptor potential 5 (TRP5), a candidate for the receptor activated calcium channels (RACCs), could be a potential target of EP1 in the process of Ca^{2+} influx (Tabata et al., 2002). EP1 receptor can couple also to Gαi/o, thus promoting PI3K activation (Ji et al., 2010)..

b. EP2 receptor

EP2 receptor is coupled to Gαs which activates adenylate cyclase leading to an elevation in intracellular cAMP levels, and consequently PKA activation. Active PKA phosphorylates different targets including certain transcription factors like the cAMP-responsive element binding protein (CREB). EP2 activates also β-catenin via PKA (Fujino et al., 2002; O'Callaghan and Houston, 2015) leading to an increase in the transcription of several genes including c-myc, cyclin D1 and VEGF, which are known to be involved in cancer. EP2-mediated signaling in endothelial cells was also implicated in the regulation of cell survival and motility, hence provoking tumor angiogenesis (Kamiyama et al., 2006).

c. EP3 receptor

Eight EP3 receptor isoforms were identified in humans. They result from alternative splicing, and differ in their C-terminal tail and in the signaling pathways to which they are associated (Dey et al., 2006a; Kotelevets et al., 2007). Isoforms of EP3 receptors are coupled to different G-proteins: Gαi, Gαs, Gαq and Gα12/13 (An et al., 1994;

Breyer et al., 2001; Israel and Regan, 2009) with the majority (EP3-I, EP3-II, EP3-III, EP3-IV, EP3-e and EP3-f) acting through Gαi, causing the inhibition of cAMP production and the activation of Ras/Raf and MAPK pathway. Some isoforms such as EP3-I, EP3-II, and EP3-III may also contribute to an increase in the levels of IP3 and intracellular calcium (Kotani et al., 1995; Kotelevets et al., 2007; Schmid et al., 1995; Woodward et al., 2011). Three EP3 receptor isoforms were identified in mice: EP3α, β, and γ (Hatae et al., 2002a; Irie et al., 1993; Sugimoto et al., 1993). EP3γ may be coupled to Gs, and thus stimulates adenylyl cyclase, and EP3β may also increase cAMP levels through the Gq/PLC/Ca^{2+} pathway in a lipid rafts dependent manner (Hatae et al., 2002b; Irie et al., 1993; Yamaoka et al., 2009). All these three isoforms were reported to induce calcium mobilization through Gβγ of the Gi/o protein, after activation of PLC β (Hatae et al., 2002a; Irie et al., 1994).

d. EP4 receptor

EP4 receptor is special in having a relatively longer cytoplasmic tail in addition to 25 amino acids inserted in its third intracellular loop. Both of these features are implicated in the coupling of EP4 receptor to G proteins. It is also different from other receptors in undergoing rapid internalization in response to PGE2 (Regan, 2003b). Like EP2, EP4 receptor activates adenylate cyclase through Gαs, yet this activation seems to be less efficient in EP4 compared to EP2. It acts also through Gαi, and activates phosphoinositide-3-kinase (PI3K) (Dey et al., 2006a; Fujino and Regan, 2006; Ichikawa et al., 2010b; Regan, 2003b), through which it may activate T cell factor (TCF)-β-catenin (Fujino et al., 2002). The EP4-mediated PI3K activation causes in addition the phosphorylation and activation of ERK1/2, which in turn stimulates the early growth response factor-1 (EGR-1) that regulates

many genes implicated in inflammation and proliferation, such as TNF-α, PGE2 synthase, and cyclin D1 (Fujino et al., 2003b).

E. Protein Kinase A

Protein Kinase A (PKA) or cAMP-dependent protein kinase is a member of AGC kinases (Taylor et al., 2012). It is a holoenzyme made up of two regulatory (R) subunits and two catalytic (C) subunits (Krebs and Beavo, 1979). It becomes active when two cAMP molecules bind to each R subunit, promoting a conformational change and the release of the two catalytic subunits which phosphorylate the corresponding downstream substrates (Welch et al., 2010). The catalytic subunits belong to the family of Ser/Thr protein kinases. PKA was shown to have more than 250 substrates (Greenwald and Saucerman, 2011), and is involved in several cellular processes, including metabolism regulation and gene transcription, as well as cell division, growth, and differentiation (Skalhegg and Tasken, 2000).

Two types of the R subunit have been identified, RI and RII, each existing in two isoforms: RIα and RIβ, and RIIα and RIIβ. Accordingly, PKA could exist as PKA type I (containing RI) or PKA type II (containing RII). RI and RII have different localizations and levels of expression, and it is thought that they have variable sensitivities for cAMP (Cadd and McKnight, 1989; Corbin et al., 1977; Corbin et al., 1978). RIα and RIIα are expressed ubiquitously (Clegg et al., 1988; Lee et al., 1983; Scott et al., 1987), while RIβ is mainly present in the brain (Cadd and McKnight, 1989), and RIIβ in the brain, fat, endocrine tissues, and reproductive organs (Jahnsen et al., 1986; Tasken et al., 1997).

Three isoforms exist for the C subunit: Cα, Cβ, and Cγ (Scott et al., 1990; Tasken et al., 1993). Cα isoform has 3 splice variants ($C\alpha_{1-3}$), with $C\alpha_1$ ubiquitously expressed, and $C\alpha_2$ expressed mainly in sperm cells, whereas $C\alpha_3$ expression still needs to be defined (Turnham and Scott, 2016). Cβ has several splice variants and is extensively expressed in various tissues. Cγ was shown to be present mainly in testis (Søberg et al., 2013).

The R subunit of PKAs contains a conserved dimerization and docking domain (D/D domain) at its N-terminus. The D/D domain is important for the dimerization of the two R subunits as well as for the binding of A-kinase anchoring proteins (AKAP). The D/D segments consist of an anti-parallel helical bundle forming a hydrophobic platform for the binding of AKAP (Banky et al., 1998; Gold et al., 2006; Kinderman et al., 2006). A common characteristic of AKAPs is a 14-18-residue amphipathic α-helix, which allows the binding to PKA. All R subunit isoforms have this conserved binding segment (Alto et al., 2003; Burgers et al., 2015; Dema et al., 2015; Kinderman et al., 2006; Skroblin et al., 2010). Binary interactions of PKA with AKAPs promote the targeting of PKA holoenzymes to specific intracellular micro-domains and determine the second messengers activated (Langeberg and Scott, 2015; Skroblin et al., 2010; Taskén and Aandahl, 2004; Wong and Scott, 2004).

cAMP is considered one of the most important transient and diffusible second messengers, acting downstream G-protein coupled receptors (GPCRs) and implicated in signal transduction through several pathways (Gancedo, 2013) involving PKA (Langeberg and Scott, 2015; Taylor et al., 2012; Taylor et al., 2008). The conversion of ATP into cAMP is mediated by adenylyl cyclase (AC) which can be activated or inhibited by

respectively the ($G_{\alpha s}$) or the ($G_{\alpha i}$) of the G-protein (Neves et al., 2002; O'hayre et al., 2013; Pierce et al., 2002; Stefan et al., 2011; Tobin et al., 2008). The cellular diffusion of cAMP is under tight spatio-temporal control by the cyclic nucleotide specific phosphodiesterase (PDE) that mediates its degradation, and stops its signaling (Baillie, 2009; Houslay et al., 2007; Kaupp and Seifert, 2002; Seino and Shibasaki, 2005).

PKA activation could also be cAMP-independent, through an involvement of the nuclear factor κB (NF-κB). In mammalian cells, NF-κB is controlled by three inhibitor isoforms IκBα, β, and ε (Hoffmann et al., 2002) that complex with NF-κB. The NF-κB:IκB complex interacts with the C subunit of PKA (and not with the R subunits) through a sequence motif at the N-terminus, where in basal conditions, the NF-κB:IκBα/β complex masks the ATP binding site and stabilizes the C subunit in its inactive form. The phosphorylation and degradation of the NF-kB:IkBα/β complex under the effect of upstream vasoactive peptides, releases the C subunit and causes its activation (Dulin et al., 2001; Zhong et al., 1997). Another cAMP-independent mechanism of PKA activation involves the Smad proteins. Activation of the transforming growth factor-beta (TGF-β) receptor is followed by the phosphorylation of two members of SMAD proteins family; Smad2 and Smad3. These two phosphorylated proteins interact with Smad4 to form heterodimeric complexes. The active Smad complex attaches to the R subunit of PKA leading to C subunit activation, and phosphorylation of PKA targets (Yang et al., 2008; Zhang et al., 2004). Some studies established a role for the Ubiquitin Proteasome System (UPS) in influencing PKA stability, activity, and signal transduction. The mammalian RING-H2 protein “praja2” complexes with PKA and causes its recruitment to different

subcellular localizations including the organelles, the perinuclear fraction, and the cell membrane. The activation of PKA leads to the phosphorylation of praja2, which targets the R subunit into proteolysis by UPS. Consequently, the R:PKAc ratio decreases, maintaining an increased phosphorylation of PKA targets (Lignitto et al., 2013; Lignitto et al., 2011; Rinaldi et al., 2015; Yu et al., 2002). UPS was also proved to be implicated in regulating the ubiquitylation and proteasomal degradation of PDEs causing elevated cAMP levels, thereby, PKA activation (Rinaldi et al., 2015; Zhu et al., 2010).

PKA activity could be downregulated by different effectors including the endogenous heat-stable PKA inhibitor peptide (PKI) (Scott et al., 1985). Interestingly, the export of the C subunit from the nucleus is mediated through its nuclear export signal, which is unmasked when PKI binds the subunit. However, PKI ultimately causes PKA inactivation through competing with its substrates, thus inhibiting the PKA nuclear functions (Dalton and Dewey, 2006; Dalton et al., 2005; Wen et al., 1995a; Wen et al., 1995b). Recent studies showed that one member of the Rho GTPase-activating protein (RhoGAP) family, ARHGAP36, is capable of direct binding to the PKA catalytic subunit, causing its ubiquitin-mediated lysosomal degradation, and downregulating PKA signaling (Eccles et al., 2016). Moreover, the A-kinase interacting protein 1 (AKIP1), which is highly conserved among mammals, traps the C subunit in the nucleus, through the nuclear localization signal (NLS) at the N-terminus of AKIP1 (Sastri et al., 2005).

Calcium is also involved in the regulation of PKA activity. By stimulating various PDEs it decreases the level of cAMP and downregulates PKA (Bender and Beavo, 2006). Calcium could also function as a cofactor for PKA phosphorylation reactions, along with

other cations such as Mg^{2+}. Calcium apparently reduces the speed of transfer of the phosphoryl group to the protein substrates, while Mg^{2+} exerts a positive effect in this context (Knape et al., 2015).

F. Phosphoinositide-3-Kinase

Phosphoinositide-3-Kinases (PI3Ks) represent a large group of enzymes that mediate the transfer of the γ-phosphate group of ATP to 3′OH group of phosphatidylinositols (PtdIns) present on the plasma membrane (Kaplan et al., 1987; Whitman et al., 1985; Wymann and Pirola, 1998). Based on their structure and substrate specificity, eight PI3K isozymes were identified and grouped into three classes (PI3K I-III) (Jean and Kiger, 2014; Vanhaesebroeck et al., 2010).

PI3Ks class I (~200 kDa) are heterodimers comprised of a catalytic subunit (110-120 kDa) and an adaptor subunit (50-100 kDa), and are capable of phosphorylating phosphatidylinositol (PtdIns), phosphatidylinositol-4-phosphate (PtdIns-4-P), and phosphatidylinositol-4,5bisphosphate (PtdIns-4,5-P2), with a preference for PtdIns-4,5-P2 under normal physiological conditions (Hawkins et al., 1992; Stephens et al., 1991). Class I PI3Ks comprises two subclasses; class IA (PI3Kα, PI3Kβ, and PI3Kδ) and class IB (PI3Kγ) which are classified according to their mode of regulation (El Sheikh et al., 2003).

PI3Ks class II (170-210 kDa) have a typical C-terminal C2 homology domain, and have a limited specificity for PtdIns and PtdIns 4-P. They are subdivided into three groups: PI3K-C2α, PI3K-C2β, and PI3K-C2γ (Martini et al., 2014). The adaptor subunits of class II PI3Ks are not identified yet, and their mode of activation is still unclear.

Class III PI3Ks phosphorylate only PtdIns, and are considered homologues of *S. cerevisiae* vacuolar protein sorting mutant Vps34p whose activity relies on Vps15p protein Ser/Thr kinase, which causes the recruitment of phosphatidylinositol kinase to the Golgi (De Camilli et al., 1996; Herman and Emr, 1990; Schu et al., 1993a).

When PI3Ks are stimulated by extracellular agonists, they are translocated to the plasma membrane in order to interact with their lipid substrates.

Class I PI3Ks are classified into two groups A and B according to the adaptor proteins implicated in the activation mechanism: class IA members are usually associated with p85 and phosphorylate tyrosine motifs, while class IB (PI3Kγ) members are associated with trimeric G proteins and p101 protein (Wymann and Pirola, 1998).

Class IA PI3Ks are constitutively associated with a regulatory subunit (50-85 kDa), typically p85, which is formed of a Src homology 3 (SH3) domain at the N-terminus, a breakpoint-cluster-region homology (BH) domain bordered by two proline-rich segments, and two SH2 domains separated by an inter-SH2 (iSH2) domain at the C-terminus. The attachment of the catalytic subunit to p85 is carried out through the SH2 domain (Escobedo et al., 1991; Otsu et al., 1991; Skolnik et al., 1991). Various mechanisms induce the activation of class IA PI3Ks. One involves interaction of the SH2 domain of p85 with pYXXM motives of receptor tyrosine kinases and the translocation of the enzyme to the plasma membrane (van der Geer et al., 1994; Wymann and Pirola, 1998). Another mode of activation includes the binding of the catalytic subunit to active Ras (Kodaki et al., 1994; Rodriguez-Viciana et al., 1994; Vanhaesebroeck et al., 1997). Interaction of SH3 domain of p85 with the proline-rich segments of some proteins such as Shc, Cbl, dynamin,

(Dombrosky-Ferlan and Corey, 1997; Gout et al., 1993; Harrison-Findik et al., 1995; Hunter et al., 1997; Soltoff and Cantley, 1996), Lyn, Fyn, Grb2, v-Src, Abl, and Lck induces the activation of class IA PI3Ks (Kapeller et al., 1994; Liu et al., 1993; Mak et al., 1996; Pleiman et al., 1994; Wang et al., 1995).

Class IB (PI3Kγ) is activated by Gi or Gq-protein coupled receptors such as m1 and m2 muscarinic receptors that activate the Akt/PKB axis via PI3Kγ (Murga et al., 1998). It is established that the binding of the released Gβγ subunits to PI3Kγ targets the latter to the plasma membrane (Leopoldt et al., 1998; Stephens et al., 1997; Stoyanov et al., 1995). Studies showed that PI3Kγ is associated at its N-terminus with a novel protein with a molecular weight of 101 kDa (p101), that is important for sensitizing p110γ for the Gβγ subunits. Other studies propose that the activation of PI3Kγ by Gβγ could happen by allosteric interaction or simply by translocating the enzyme to the membrane (Bondeva et al., 1998; KRUGMANN et al., 1997; Stephens et al., 1997).

Class II PI3Ks interact weakly through their C2 domains with phospholipids in a Ca^{2+}-independent manner (MacDougall et al., 1995), where C2 domains (necessary for the enzyme's catalytic activity (Misawa et al., 1998)) are insensitive to Ca^{2+} due to the absence of Asp residues that are essential for Ca^{2+} binding (Sutton et al., 1995).

The only known enzyme of class III PI3Ks, and the only PI3K expressed in all eukaryotes is the vacuolar protein sorting 34 "Vps34" (aka PI3K-C3) which is an archetype of class III PI3Ks expressed in *Saccharomyces cerevisiae*. It produces phosphatidylinositol-3-phosphate which regulates the intracellular vesicular transport (Abe et al., 2009; Jaber and Zong, 2013; Schu et al., 1993b). The Vps34p protein expressed in yeast is usually

associated with Vps15p protein Ser/Thr kinase which is indispensable for the activation of Vps34p and its translocation to the Golgi (Stack et al., 1995; Stack et al., 1993). Vps34p accounts for the production of all PtdIns 3-P in yeast, yet the source of production of this molecule in mammalian cells is still undetermined, but it is thought that the interaction of certain lipid receptors with GTPases and lipid-modifying enzymes would regulate protein sorting, endocytosis, and exocytosis (Wymann and Pirola, 1998).

PI3Ks have several downstream targets, one of which is the Ser/Thr kinase Akt/PKB. PtdIns(3,4)P2 binds to the PH domain of Akt/PKB causing its recruitment to the plasma membrane (Andjelković et al., 1997; Franke et al., 1997; Klippel et al., 1997) where it is phosphorylated at Thr308 and Ser473 positions by phosphoinositide-dependent kinases (PDKs) (Alessi et al., 1996; Andjelković et al., 1996), including PDK1 whose activity is modulated by the interaction of its PH domain with PtdIns(3,4,5)P3 and PtdIns(3,4)P2 (Alessi et al., 1997a; Alessi et al., 1997b; Stephens et al., 1998; Stokoe et al., 1997). PtdIns(3,4,5)P3 was shown to activate the integrin-linked kinase (ILK) which has the capacity to phosphorylate Akt/PKB on Ser473 position (Wymann and Pirola, 1998). Activation of PI3K is needed also for the GDP/GTP exchange of Rac1 (Hawkins et al., 1995). Atypical PKCs also represent targets for PI3Ks (Toker and Cantley, 1997) as well as Bruton's tyrosine kinase (Btk) which is activated by PtdIns(3,4,5)P3 and Src protein tyrosine kinase (Li et al., 1997; Rameh et al., 1997) and is considered important for the normal function and development of B lymphocytes (de Weers et al., 1994; Hendriks et al., 1996). Phospholipase Cγ (PLCγ) is also activated by PtdIns(3,4,5)P3, leading to its translocation to the membrane where their substrates are present or where growth factor

receptors are present (specifically to their phosphorylated tyrosine motives) (James and Downes, 1997; Valius and Kazlauskas, 1993).

G. P38-Mitogen Activated Protein Kinase

The p38-mitogen activated protein kinase or p38 MAPK is a 38 kDa protein that belongs to the MAPK subfamily, and is the major kinase involved in stress signaling (Freshney et al., 1994; Han et al., 1994; Wang et al., 2010). Its activation was linked to various cellular and physiological processes such as inflammation, cell growth and differentiation, as well as cell death in several tissues (Liu et al., 2005).

P38MAPK has a catalytic site that falls between the N-terminus and C-terminus, which are connected together by a hinge region and by the L16 loop in the C-terminal, which folds around the N-terminal end and controls the link between the rigid domains (Wang et al., 1998). Substrates and activators bind p38 MAPK through docking domains composed of two regions; the CD region and the ED region (Tanoue et al., 2000; Wang et al., 1998). A flexible activation loop shielding the active site contains two particular Thr^{180} and Tyr^{182}, that become phosphorylated by certain stimuli including physical stress, cytokines, endotoxin, and chemical oxidant stress. These two coupled phosphorylations induce the refolding of the activation loop, which exits the peptide binding channel, causing a change in the tertiary structure of the enzyme, facilitating ATP binding and permitting the interaction with the substrate (Diskin et al., 2007; Freshney et al., 1994; Han et al., 1994; Raingeaud et al., 1995; Rouse et al., 1994; Wilson et al., 1996b).

P38MAPK has four isoforms (α, β, γ, and δ) that have different sensitivities to pyridynylimadazole inhibitors (e.g. SB203580). The α and the β isoforms of p38 MAPK are sensitive to SB203580, and are ubiquitously expressed, however p38γ and p38δ show resistance to SB203580 due to structural differences in their ATP-binding pocket. p38γ-MAPK is expressed only in muscles, and p38δ-MAPK is expressed mainly in glomeruli and lungs. The presence of different isoforms contributes to diverse p38-MAPK signaling pathways (Gum et al., 1998; Nick et al., 2002; Wilson et al., 1996a).

Several extracellular stimuli activate p38-MAPK such as heat shock, UV light, osmotic stress, and proinflammatory cytokines like interleukin-1 (IL-1) and tumor necrosis factor alpha (TNF-α) (Sugden and Clerk, 1998). Stress signals induce the activation of p38 MAPK by MKK3 and MKK6, which are MAPK kinases (MKKs). MKKs themselves are activated through their phosphorylation by MAPK kinase kinases (MKKKs) (Cheung et al., 2003; Clark et al., 2007; Gallo and Johnson, 2002; Ge et al., 2002; Hutchison et al., 1998; Ichijo et al., 1997; Moriguchi et al., 1996; Yamaguchi et al., 1995). Some studies showed that the interaction of p38 MAPK with the non-enzymatic adaptor protein transforming growth factor-β-activated protein kinase-1 (TAK1) binding protein-1 (TAB1), helps in driving p38 autophosphorylation. TAB1 also promotes the autophosphorylation of TAK1, which activates MKK3/MKK6 (Clark et al., 2007; Ge et al., 2002). In contrast, other studies propose that TAB1 induces p38 to redistribute in the cytoplasm, thus limiting its interaction with the downstream target molecules including MAPK-activated protein kinase 2 (MAPKAPK2) (Lu et al., 2006). Moreover, the activation of T-cell antigen receptor (TCR) recruits ZAP-70, a Syk family kinase, which phosphorylates p38 on a Tyr^{323} residue,

followed by autophosphorylation and activation of p38 MAPK (Clark et al., 2007; Dong et al., 2002; Rincón and Pedraza-Alva, 2003; Rouse et al., 1994).

There are several known inhibitors of p38 MAPK. They are in general classified in two types, depending on the way they bind to p38 MAPK. The first type includes inhibitors that bind to the active site (such as SB203580); these are also known as gatekeeping inhibitors. The second type includes inhibitors that bind distantly (such as BIRB-796), and affect the binding of ATP to p38 in an indirect way (Clark et al., 2007). It is proposed that SB203580 competes with ATP and binds to the active site of both active and inactive forms of p38 (Clark et al., 2007; Wang et al., 1998). However, urea-containing p38α inhibitors, like BIRB-796, bind to a position distant from the ATP-binding pocket, and promotes the movement of the Phe^{169} residue, which in turn occupies the ATP pocket and inhibits the binding of ATP (Pargellis et al., 2002).

The classical effect of p38MAPK involves the activation and translocation of some transcription factors to the nucleus, such as AP-1 and SP-1, leading to the transcription of several downstream targets and inflammatory cytokines. p38 MAPK was shown to activate mitogen and stress activated protein kinases, like MSK1 and MSK2. These are able to amplify NF-κB signaling by phosphorylating the Ser^{276} residue of the trans-activating p65 subunit of NF-κB complex (Ono and Han, 2000; Salminen et al., 2012). In addition, p38 MAPK also activates the MAP kinase-activated protein kinases 2 and 5 (MAPKAPK2 and MAPKAPK5) that phosphorylate several proteins including heat shock proteins (Natale et al., 2004). Some studies show that p38 MAPK is able to

translocate to the nucleus and to stimulate the activity of several molecules such as p21 and p16, leading to the blockage of the cell cycle at the G2M48 and G1/S checkpoints (Bulavin et al., 2001; Krementsov et al., 2013; Thornton and Rincon, 2009). Active p38 MAPK was also shown to phosphorylate the Ser^{389} residue of the glycogen synthase kinase 3β (GSK3β), leading to its inactivation and to the maintenance of cell survival (Thornton et al., 2016).

CHAPTER III

MATERIALS AND METHODS

A. Materials

The human colonic ADENOCARCINOMA cell line (Caco2) was purchased from the American Type Culture Collection (ATCC).

Procyanidin B2 was obtained from Biochemika Int., Pakistan.

SB 202190, Adenosine-3′,5′-cyclic Monophosphorothioate Rp-Isomer Triethylammonium salt (RpcAMP), Phorbol-12-myrsitate-13-acetate (PMA), Calphostin C, and Wortmannin were all purchased from Calbiochem San Diego, USA.

SC-19220, PF-04418948, L-798106, and BGC-201531 were obtained from Cayman Chemical Company, Michigan, USA.

Luminol reagent used for Western Blot, nitrocellulose membranes, and reagents for protein assay were bought from Biorad, California, USA. Tablets of protease inhibitor cocktail were obtained from Boehringer Mannheim, Germany.

Indomethacin, Prostaglandin E2 (PGE2), ouabain, Fetal Bovine Serum (FBS), Trypsin-EDTA, Penicillin/Streptomycin (Penstrep), 10x Phosphate Buffered Saline (PBS) without magnesium and calcium, Dulbecco's Minimal Essential Medium (DMEM)

supplemented with 4500 mg/L glucose and pyridoxine HCL, 2'-O-Dibutryryladenosine 3′,5′-cyclic monophosphate sodium salt (dbcAMP), and Adenosine 5′-triphosphate disodium salt (ATP) were all purchased from Sigma, Chemical Co, St Louis Missouri, USA.

Anti-Na^+/K^+ ATPase primary antibody was purchased from EMD Millipore Corp., Burlington, MA 01803, USA.

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

B. Methods

1. Culture of Caco2 cells

Caco2 cells at passages 29 to 52 were cultured in Dulbecco's Minimal Essential Medium (DMEM) fortified with 1% penstrep (penicillin 100µg/ml, streptomycin 100µg/ml) and 10% Fetal Bovine Serum (FBS). Cells were seeded in 6-well plates, at a density of around 350,000 cells/well and incubated in a humidified incubator under optimal conditions for cell survival and division (95% O2, 5% CO2, 37°C).

2. *Treatment of Caco2 cells*

a. Effect of Procyanidin B2 on Na^+/K^+ ATPase activity

Caco2 cells were treated at a confluence of 80%-85% with 17µM Procyanidin B2 for different time periods (15 min, 30 min, 1hr, 2 hrs, and 3 hrs) to determine the optimal time for the appearance of the effect of Procyanidin B2 on Na^+/K^+ ATPase activity.

A dose response study was then conducted to study the effect of different concentrations of Procyanidin B2 (5 μM, 10 μM, 17 μM, 35 μM, and 70 μM) on the Na^+/K^+ ATPase when applied to the cells for 2 hrs, which is the optimal time determined above. An equal volume of the vehicle DMSO was added to the control.

At the end of every treatment, the cells were washed with 1ml of 1X sterile phosphate buffered saline (PBS, pH 7.4) and the plates were preserved in the deep freezer at -80ºC for later analysis and assay of the Na^+/K^+ ATPase activity.

b. Involvement of PKC

Involvement of PKC in the effect of procyanidin B2 (17μM, 2hrs) was tested using Calphostin C (50 nM, DMSO), a PKC inhibitor. Calphostin C was added to the cells, 30 minutes before Procyanidin B2. Cells were also treated with Phorbol 12-myristate 13-acetate (PMA) (1μM, DMSO), a PKC activator, for 2 hours for further confirmation.

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

The involvement of PGE2 was tested using indomethacin (100 μM, DMSO), an inhibitor of the cyclooxygenase enzymes, or by treating the cells with exogenous PGE2 (100 nM, alcohol). PGE2 was added for 2 hours, however indomethacin was added 30 minutes before Procyanidin B2.

To determine the type of PGE2 receptors involved in the effect of Procyanidin B2, cells were treated with a specific antagonist to each receptor. These are: SC-19220 (EP1 antagonist; 100 μM in DMSO), PF-04418948 (EP2 antagonist; 1 μM in DMSO), L-798106 (EP3 antagonist, 10 μM in DMSO), and BGC-201531 (EP4 antagonist; 10 μM in DMSO). All of the mentioned antagonists were added 30 minutes before Procyanidin B2.

d. Involvement of PKA

Cells were treated with RpcAMP (30 μM, water), an inhibitor of PKA, 30 minutes before Procyanidin B2, to check if PKA is a mediator. For further confirmation, cells were treated for 2 hours with dbcAMP (10 μM, water), a cell permeable cAMP analogue.

e. Involvement of PI3K and P38MAPK

The involvement of PI3K and p38MAPK was investigated by treating the cells 30 minutes before Procyanidin B2 with their respective inhibitor Wortmannin (100 nM, DMSO) and SB 202190 (50 μM).

f. Positioning the mediators involved with respect to each other

In order to position both PI3K and PKA with respect to PGE2, cells were treated with exogenous PGE2 in presence of Wortmannin (100 nM, DMSO) or RpcAMP (30 μM, water), respective inhibitors of PI3K and PKA which were added 30 minutes before PGE2 (100 nM, alcohol, 2hrs).

To determine whether PGE2 and PI3K fall upstream or downstream PKC, cells were treated with Indomethacin (inhibitor of cyclooxygenase enzymes) or Wortmannin (PI3K inhibitor) for 30 minutes. This was followed with a 2 hour-treatment with PMA (PKC activator) still in the presence of the inhibitors.

3. Sample Preparation

Treated cells were scraped in lysis buffer containing protease inhibitors (9.9 mL Histidine buffer (150 mM, pH=7.4), 100 μL Triton-X, 400 μL protease inhibitor (1 tablet in 2mL H2O). Samples were then homogenized using a PRO Homogenizer at the maximum speed (30,000 rpm) for around 25 seconds. The homogenate was then spun for 30-minutes at 20000 g and 4°C. Proteins in the supernatant were quantified using Bradford Method.

Protein concentration was then adjusted accordingly and samples were used for the Na^+/K^+ ATPase activity assay or for Western Blot analysis.

4. Na^+/K^+ ATPase Activity Assay

The protein concentration of the homogenate was adjusted to 0.5 µg/µl with histidine buffer (150 mM, pH=7.4). The homogenates (65 µL) were incubated with 2% Saponin (17 µL) for 15 minutes, followed by an additional 15-minute incubation with 13 µL of phosphatase inhibitor cocktail (500 µL of 200 mM glycerophosphate, 500 µL of 200 mM pyrophosphate, 400 µL water). These incubations were done at room temperature. Next, two sets from each sample were prepared (with 3 replicates per set) as indicated in the table below.

	Histidine (150 mM, pH=7.4)	Ion mix *	Sample homogenate	Ouabain (15 mM)	Water	ATP (30 mM)
Set 1	42 µL	30 µL	12 µL	-	8 µL	10 µL
Set 2	42 µL	30 µL	12 µL	8 µL	-	10 µL

Table.1. Assay of Na^+/K^+ ATPase activity. * The ion mix was prepared by mixing equal volumes of NaCl (1240 mM), KCl (200 mM), and $MgCl_2$ (40 mM).

Samples were incubated with ATP as mentioned above at 37°C for 15 minutes. The reaction was stopped by adding 10 µL of 50% Tricholoroacetic acid, followed by a 5-minute centrifugation process at 16.000 xg. In a 96-well microplate, 90 µL of the obtained supernatants were mixed with 80 µL of a ferrous sulfate molybdate solution (for 1 mL of this solution 50 mg of ferrous sulfate were mixed with 100 µL ammonium molybdate (100

g/L in 10N H_2SO_4) and 900 μL water). The intensity of the resulting blue color increases with an increase in the concentration of liberated inorganic phosphate. The latter was measured colorimetrically, using a microplate reader at λ=750 nm.

5. Western Blot Analysis

Forty micrograms from each sample homogenate were loaded and run on 8% SDS polyacrylamide gel and then transferred to a nitrocellulose membrane. The membranes were blocked for 40 minutes in PBS (1L 1x PBS, 1 mL Tween 20, and 10 mL 0.1% sodium azide), and then incubated at 4°C overnight with anti-Na^+/K^+ ATPase primary antibody. This was followed by an incubation with HRP-conjugated anti-mouse secondary antibodies for 1 hour at room temperature. Bands were visualized by chemiluminescence using ChemiDoc^{TM}MP after adding 1 mL Clarity ECL Substrate.

6. Statistical Analysis

The data were analyzed for statistical significance by a one-way analysis of variance followed by a Tukey-Kramer multiple comparison test, using the GraphPad InStat 3 software. Data are reported as means ± SEM.

CHAPTER IV

RESULTS

A. Time response study on the effect of Procyanidin B2 on the Na^+/K^+ ATPase activity in Caco-2 cells

Caco-2 cells were treated with Procyanidin B2 at a concentration of 17 µM for different time periods. The greatest increase in the activity of the ATPase was observed at 120 minutes, with an increase of 140% compared to the control (Fig.1).

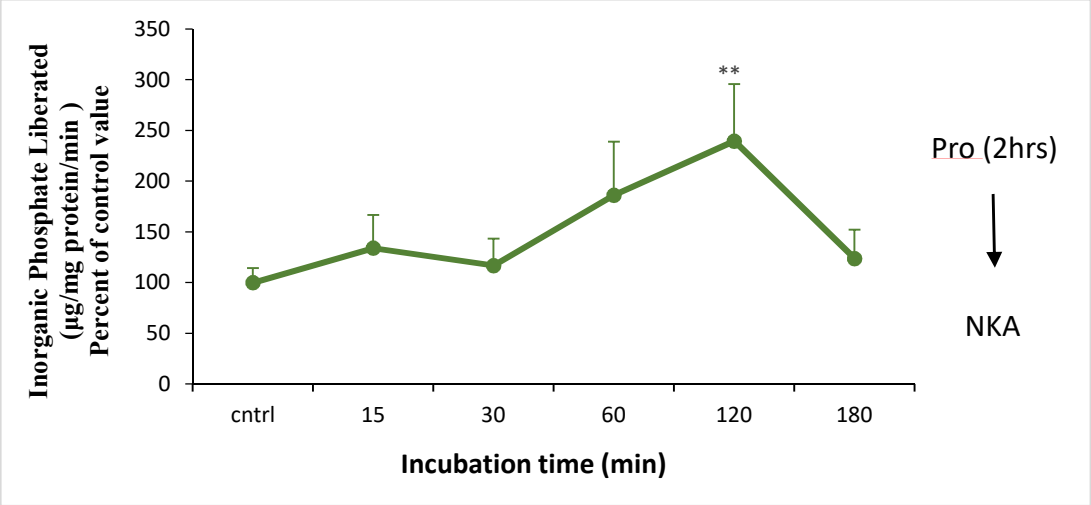

Fig.1. The maximal effect of 17 µM Procyanidin B2 was manifested at 120 min. The values are reported as means ± SEM of 3 readings. (**) significantly different from the control at $p<0.01$ after performing ANOVA followed by Tukey test. Pro=Procyanidin B2, NKA= Na^+/K^+ ATPase.

B. Dose response study on the effect of Procyanidin B2 on the Na^+/K^+ ATPase activity in Caco-2 cells

Caco-2 cells were treated for 120 minutes with different concentrations of Procyanidin B2. The maximal stimulation of the Na^+/K^+ ATPase was observed at 17 µM, with an activity of around 200% compared to that of the control (Fig.2).

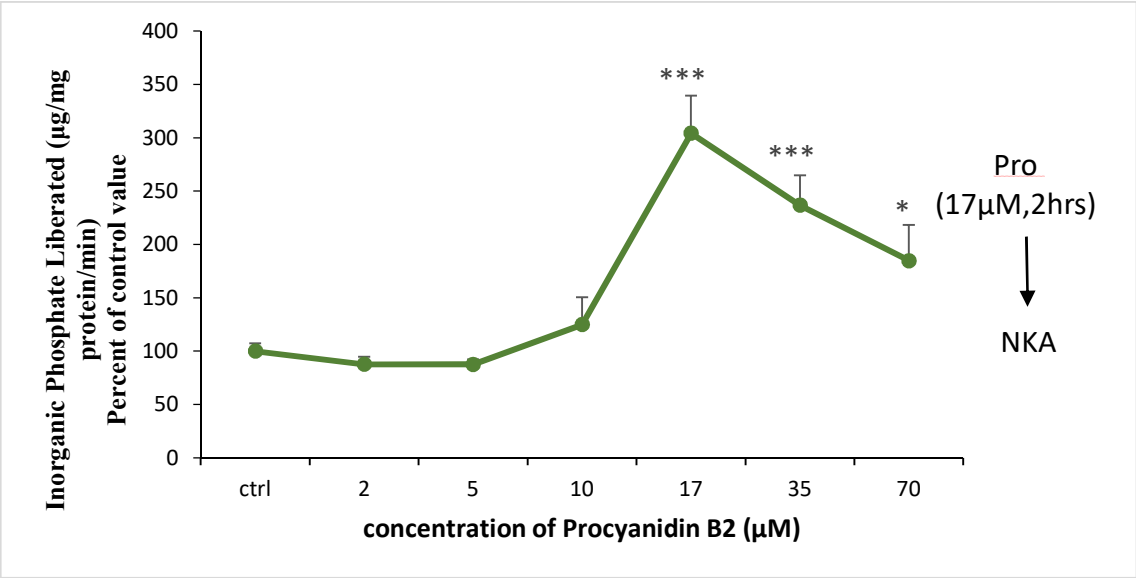

Fig.2. Procyanidin B2 induced a maximal increase in the Na^+/K^+ ATPase at a concentration of 17 µM. The values reported are means ± SEM of 3 readings. Statistical significance was tested by ANOVA and Tukey Kramer test. (***) significantly different from the control at $p<0.001$. (*) significantly different from the control at $p<0.05$. Pro=Procyanidin B2, NKA= Na^+/K^+ ATPase.

C. Involvement of PGE2 in the effect exerted by Procyanidin B2 on the Na^+/K^+ ATPase and identification of the EP receptor involved

In order to test for the involvement of PGE2 in the signaling pathway mediated by Procyanidin B2, Caco-2 cells were treated with indomethacin, an inhibitor of cyclooxygenase enzymes needed for PGE2 synthesis. In the presence of indomethacin, the effect of Procyanidin B2 was abolished. Treatment of Caco-2 cells with exogenous PGE2 mimicked the effect of Procyanidin B2 and stimulated the Na^+/K^+ ATPase (Figs.3 and 4).

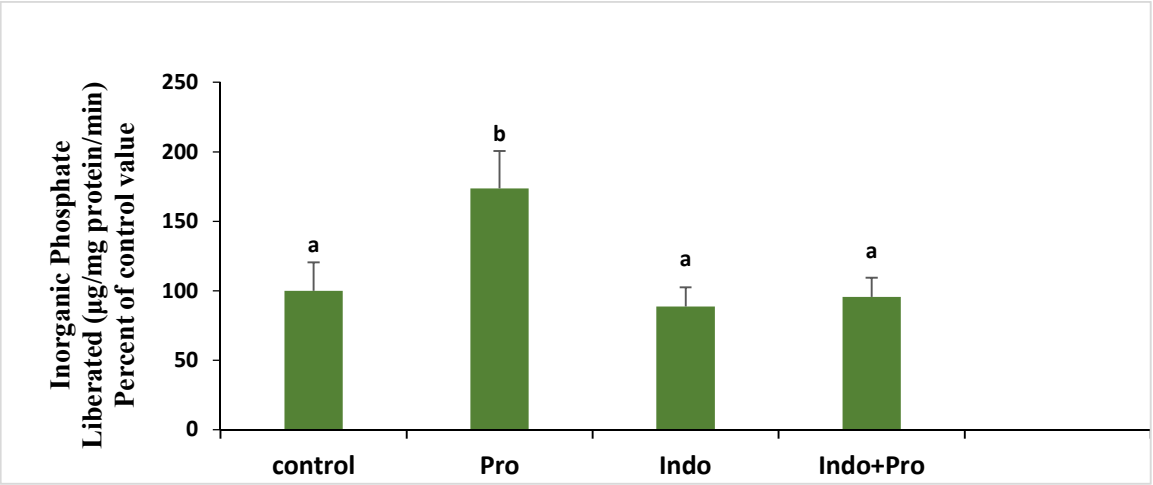

Fig.3. PGE2 is involved in the activation of the Na^+/K^+ ATPase by Procyanidin B2. The stimulation observed in the presence of Procyanidin B2 (17µM, 2hrs) disappeared in the presence of Indomethacin (100 µM,). The values are reported as means ± SEM of 3 readings. The bars that show different letters are significantly different from each other at $p<0.05$ as revealed by ANOVA followed by Tukey test. Indo=Indomethacin, Pro=Procyanidin B2.

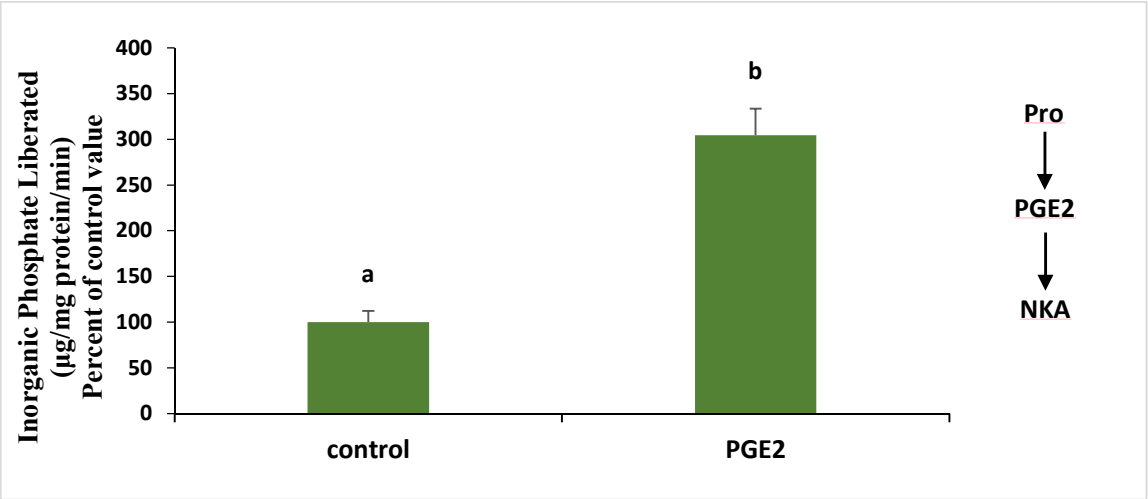

Fig.4. PGE2 activates the Na^+/K^+ ATPase. Exogenous PGE2 (100 nM, 2hrs) mimicked the stimulatory effect exerted by Procyanidin B2. The values are reported as means ± SEM of 3 readings. The bars that show different letters are significantly different at $p<0.05$ after performing ANOVA followed by Tukey test. Pro=Procyanidin B2, NKA= Na^+/K^+ ATPase.

PGE2 activates several intracellular signaling pathways by binding to four types of EP receptors (EP_{1-4}). In order to determine the type of EP receptor mediating the effect of Procyanidin B2 on the Na^+/K^+ ATPase, each EP receptor was inhibited with its specific blocker. The effect of Procyanidin B2 persisted in the presence of SC-19220, PF-04418948, and L-798106, respective blockers of EP1, EP2, and EP3, but was abolished in the presence of BGC-201531, an EP4 blocker (Figures 5,6,7, and 8).

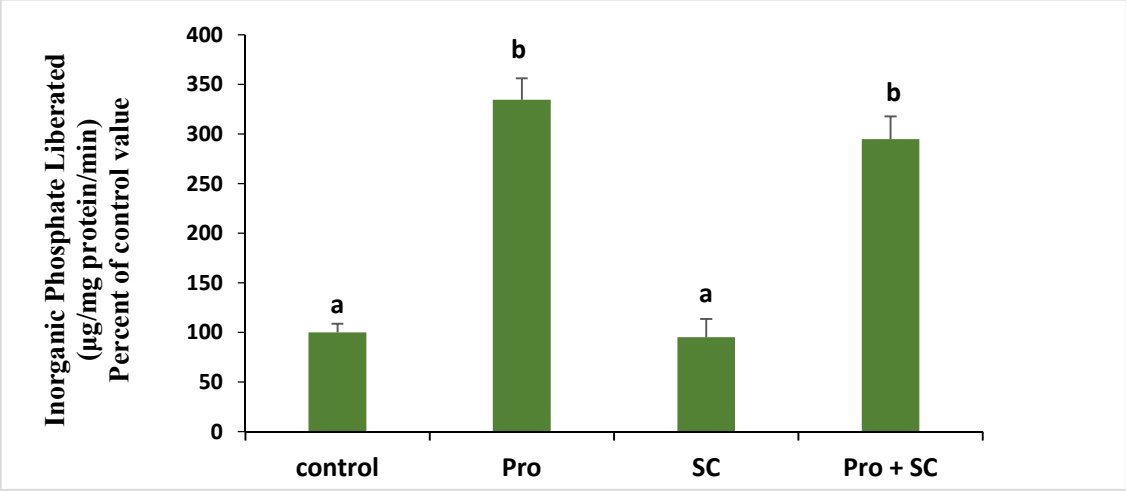

Fig.5. The effect of Procyanidin B2 persisted in the presence of SC-19220 (100 µM in DMSO), an EP1 blocker. The values are reported as means ± SEM of 3 readings. The bars with different letters are significantly different at $P<0.05$ as determined by ANOVA followed by Tukey test. SC= SC-19220, Pro=Procaynidin B2.

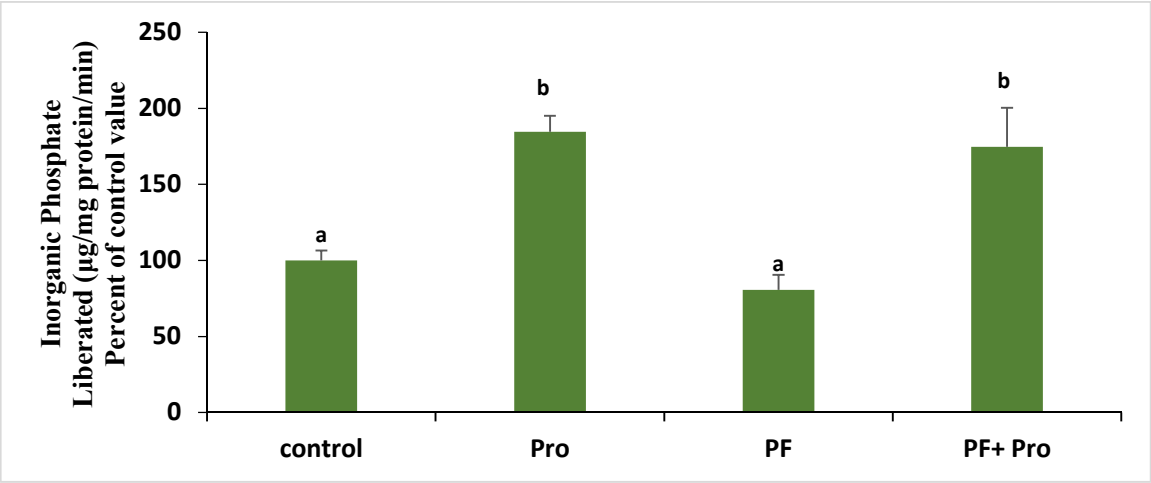

Fig.6. The effect of Procyanidin B2 was maintained in the presence of PF-04418948 (1 µM, DMSO), a blocker of EP2 receptors. The values are reported as means ± SEM of 3 readings. Significant differences were tested by ANOVA followed by Tukey test. Bars with different letters are significantly different from each other at P<0.05. PF= PF-04418948, Pro=Procyanidin B2.

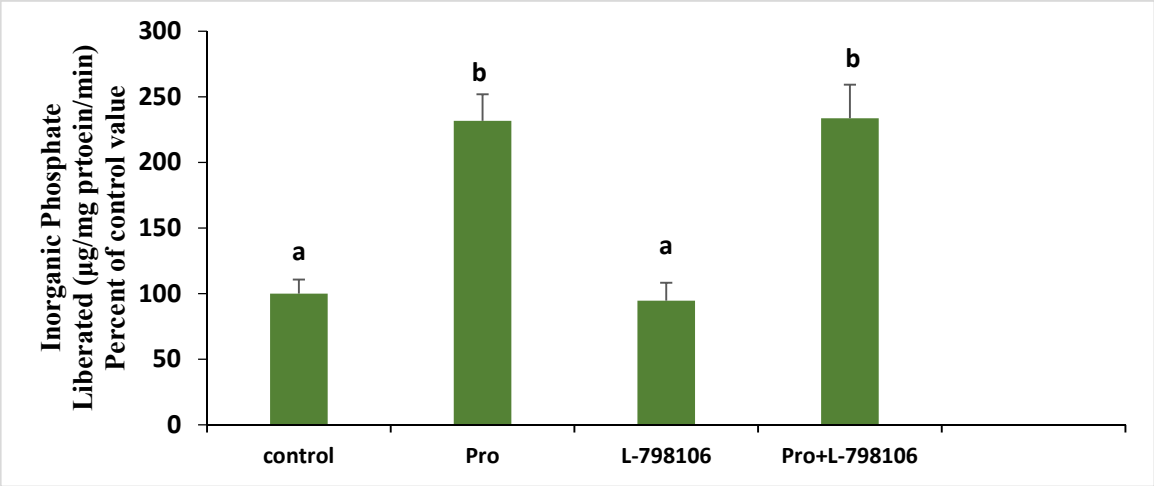

Fig.7 The effect of Procyanidin B2 was still apparent in the presence of L-798106 (10 µM, DMSO), a blocker of EP3 receptors. The values are reported as means ± SEM of 3 readings. Bars with different letters are significantly different from each other at P<0.05. Significant differences were tested by ANOVA followed by Tukey test. Pro=Procyanidin B2.

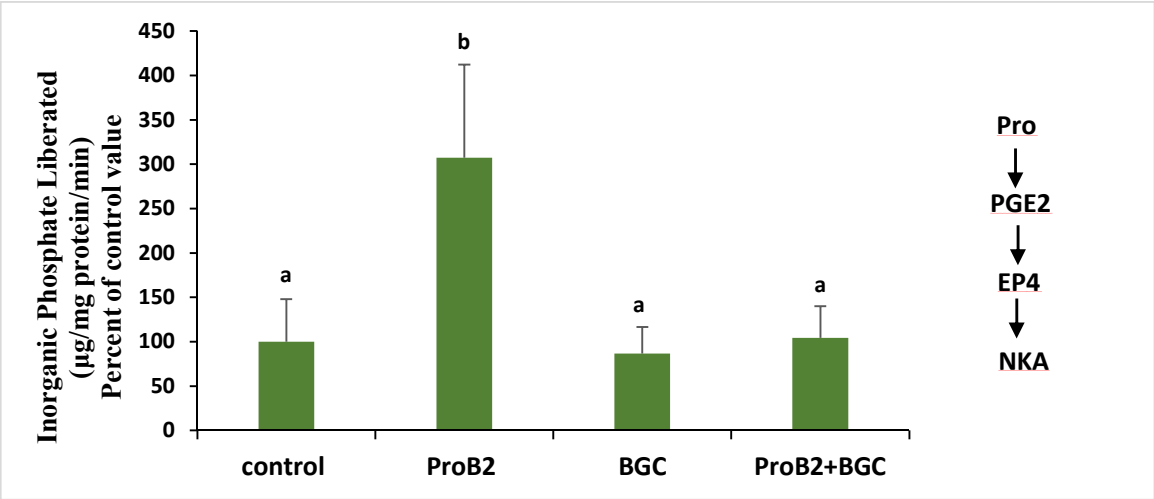

Fig.8 The effect of Procyanidin B2 was abolished in presence of BGC-201531 (10 µM, DMSO), an EP4 antagonist. The values are reported as means ± SEM of 3 readings. Bars with different letters are significantly different at $P<0.05$ as revealed by ANOVA followed by Tukey test. BGC= BGC-201531. ProB2/Pro=Procyanidin B2, NKA= Na^+/K^+ ATPase.

D. Involvement of PKC in the effect of Procyanidin B2 on the Na^+/K^+ ATPase

The involvement of PKC was investigated by treating Caco-2 cells with Calphostin C, a PKC inhibitor. In its presence, the stimulatory effect of Procyanidin B2 did not appear. Treatment with Phorbol-12-myrsitate-13-acetate (PMA), an activator of PKC, showed a similar stimulation to that observed with Procyanidin B2 (Fig.9).

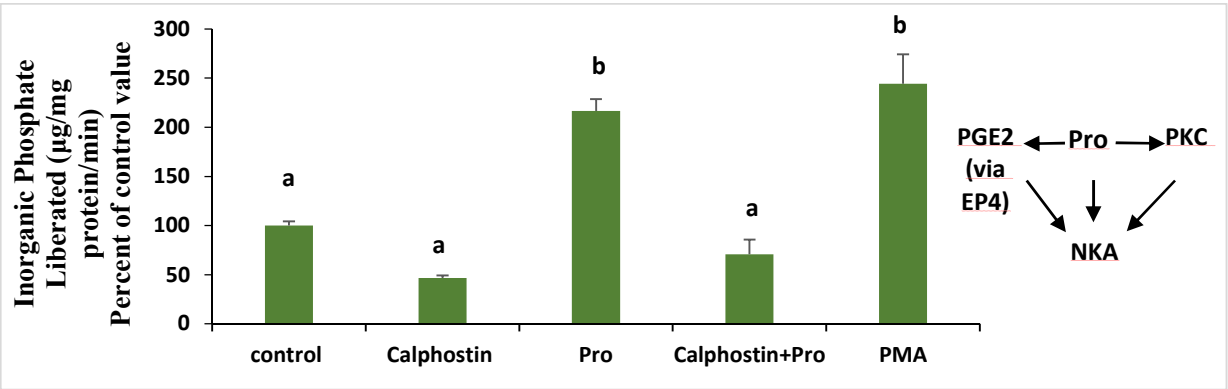

Fig.9. PKC is a mediator in the activation of the Na^+/K^+ ATPase by Procyanidin B2. The stimulation of Procyanidin B2 (17 µM, DMSO) was not observed in the simultaneous

presence of Calphostin C (50 nM, DMSO), whereas PMA (1µM, DMSO) induced an increase in the ATPase activity. The values are reported as means ± SEM of 3 readings. Bars not sharing a common letter are significantly different from each other at P<0.05 as revealed by ANOVA followed by a Tukey test. Pro=Procyanidin B2, NKA= Na^+/K^+ ATPase.

E. Involvement of PKA in the effect of Procyanidin B2 on the Na^+/K^+ ATPase

The effect of Procyanidin B2 on the Na^+/K^+ ATPase was eliminated in the presence of RpcAMP, a PKA inhibitor. A similar effect to Procyanidin B2 was observed in the presence of dbcAMP a cell permeable cAMP analogue (Fig.10).

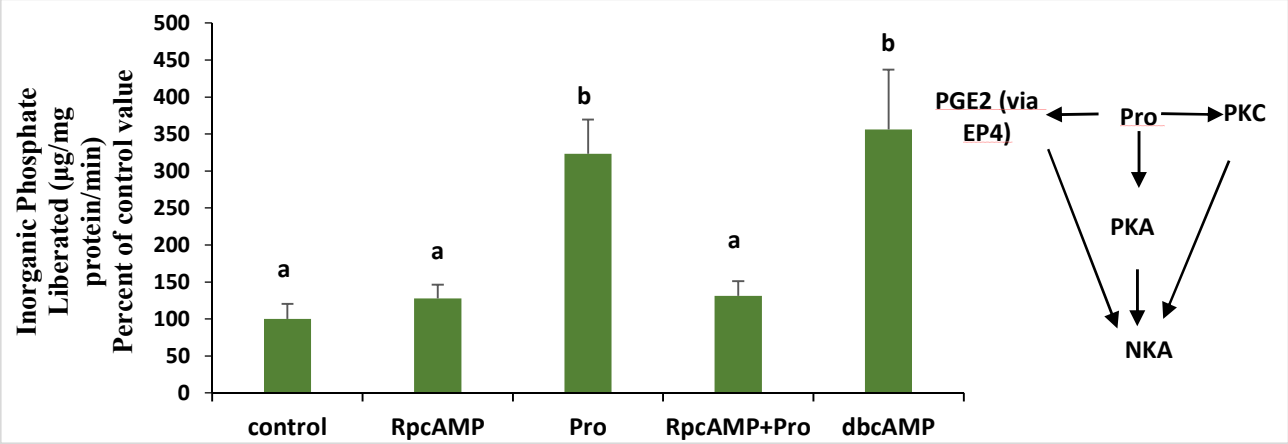

Fig.10. The effect of Procyanidin B2 (17 µM, 2hrs) disappeared in the presence of RpcAMP (30 µM), and imitated by dbcAMP (10 µM). The values are means ± SEM of 3 readings. Bars not sharing a common superscript are significantly different from each other at P<0.05 as revealed by ANOVA followed by Tukey test. Pro=Procyanidin B2, NKA= Na^+/K^+ ATPase.

F. Involvement of PI3K in the effect of Procyanidin B2 on the activity of the Na^+/K^+ ATPase

PI3K is one of the molecules known to modulate the activity of the Na^+/K^+ ATPase. Thus, its involvement in the effect of Procyanidin B2 was tested by treating Caco-

2 cells with wortmannin, a PI3K inhibitor. The stimulation of the Na^+/K^+ ATPase observed with Procyanidin B2 disappeared in the presence of wortmannin (Fig.11).

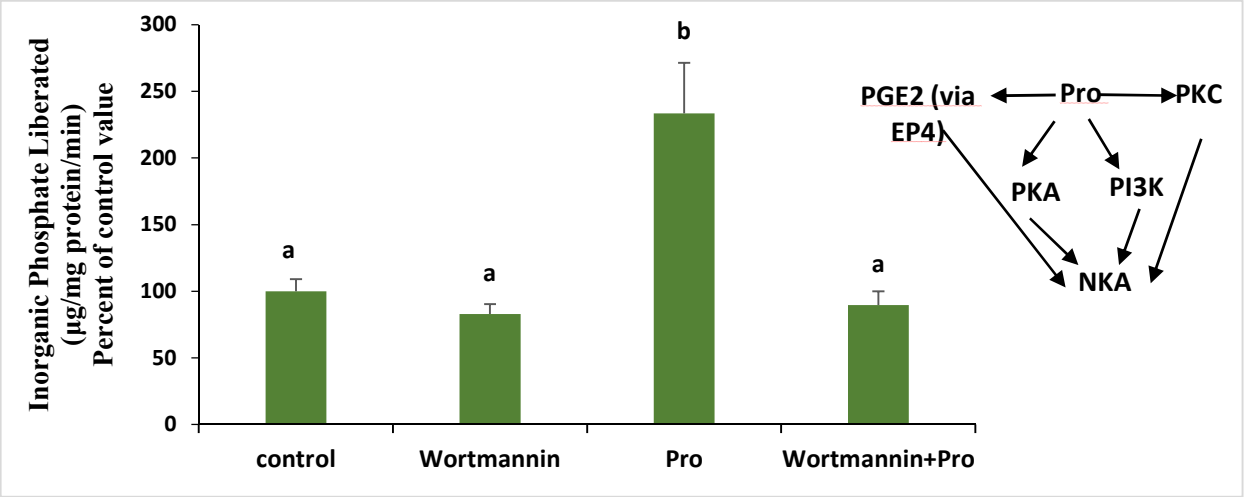

Fig.11. PI3K is involved in the effect of Procyanidin B2 on the Na^+/K^+ ATPase. Procyanidin B2 (17 µM, 2hrs) had no effect on the activity of the ATPase in the simultaneous presence of wortmannin (100 nM, DMSO). Values are means ± SEM of 3 readings. Significant differences were tested by ANOVA followed by Tukey test. Bars with different letters are significantly different from each other at $P<0.05$. Pro=Procyanidin B2, NKA= Na^+/K^+ ATPase.

G. Positioning PGE2 with respect to PKC

The stimulatory effect of PMA, a PKC activator, on the ATPase was not observed in presence of indomethacin, an inhibitor of COX enzymes (Fig.12), indicating that PKC is upstream PGE2.

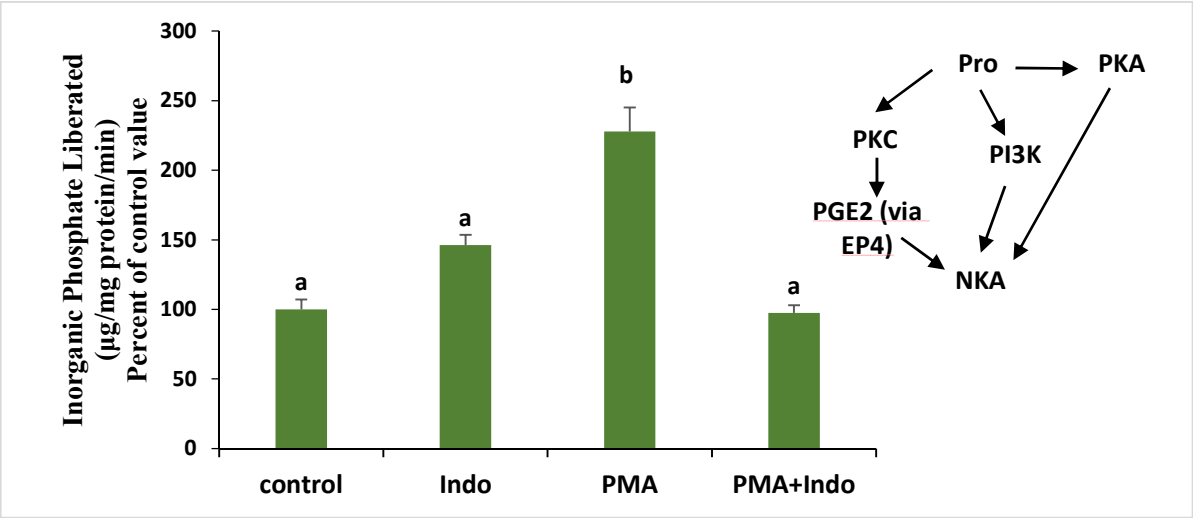

Fig.12. PKC is upstream PGE2. The values are reported as means ± SEM of 3 readings. Bars with different letters are significantly different at P<0.05 after performing ANOVA followed by Tukey test. Indo=Indomethacin, NKA= Na^+/K^+ ATPase, Pro= Procyanidin B2.

H. Positioning PGE2 with respect to PI3K

An increase in the activity of the ATPase was still observed when Caco-2 cells were treated with PGE2 in the presence of wortmannin, a PI3K inhibitor (Fig.13), indicating that PI3K is upstream PGE2.

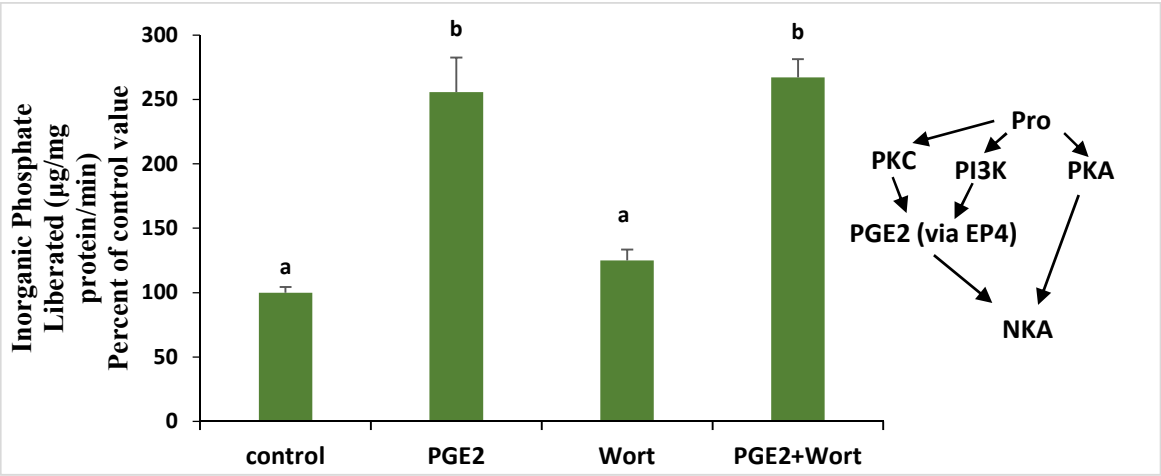

Fig.13. PI3K is upstream PGE2. Values are means ± SEM of 3 readings. Bars not sharing a common superscript are significantly different from each other at P<0.05. Statistical

significance was tested by ANOVA followed by Tukey test. Wort=Wortmannin, NKA= Na^+/K^+ ATPase, Pro= Procyanidin B2.

I. Positioning of PKC with respect to PI3K

The stimulatory effect of PMA on the Na^+/K^+ ATPase was still observed in presence of wortmannin, a PI3K inhibitor (Fig.14), thus, PI3K is upstream PKC.

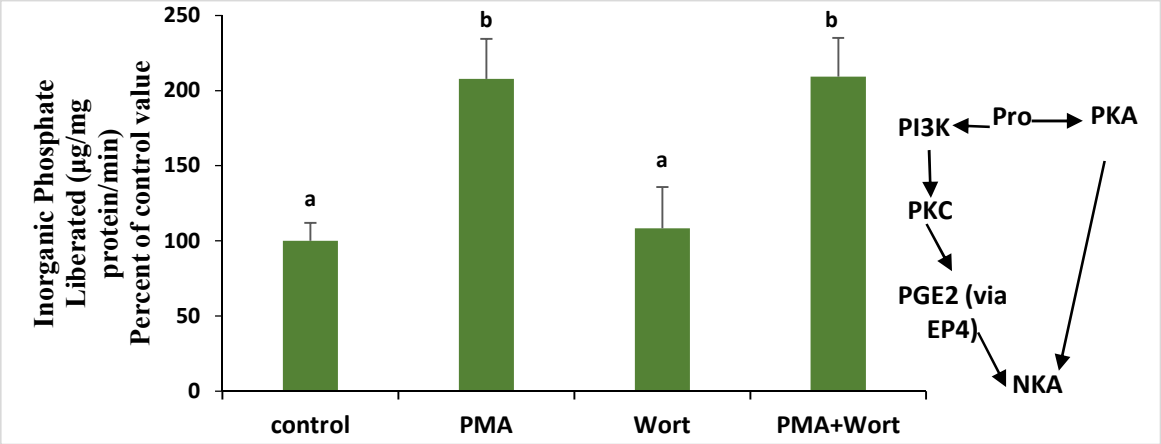

Fig.14. PI3K is upstream PKC. The values are means ± SEM of 3 readings. The bars that show a different letter are significantly different at $P<0.05$. Significant differences were tested by ANOVA followed by Tukey test. Wort=Wortmannin, NKA= Na^+/K^+ ATPase, Pro= Procyanidin B2.

J. Positioning PKA with respect to PGE2

To locate PKA with respect to PGE2, Caco-2 cells were treated with exogenous PGE2 in the presence of RpcAMP, a PKA inhibitor. The effect of PGE2 disappeared in the presence of RpcAMP (Fig.15), therefore, PKA is downstream PGE2.

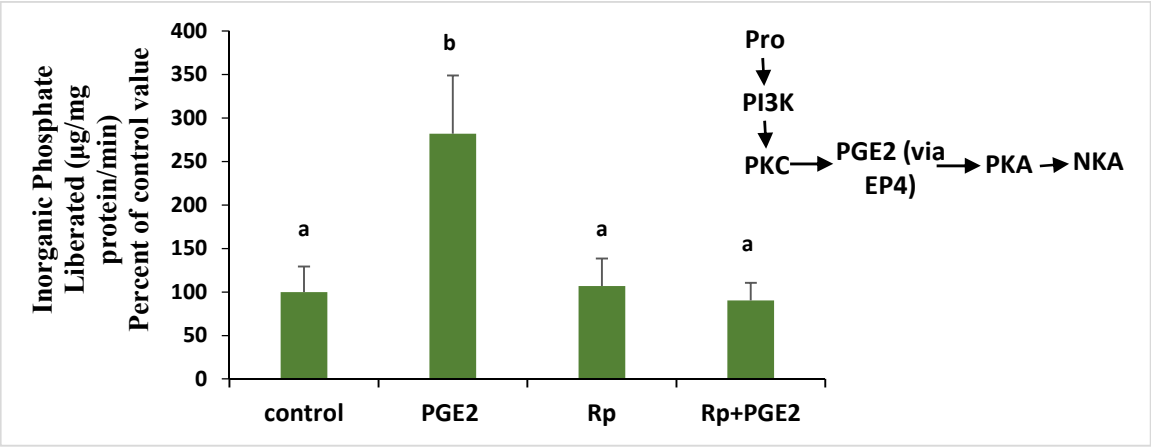

Fig.15. PKA is downstream PGE2. Values are means ± SEM of 3 readings. Bars not sharing a common letter are significantly different at $P<0.05$ as revealed by ANOVA followed by Tukey test. Rp=RpcAMP, NKA= Na^+/K^+ ATPase, Pro= Procyanidin B2.

K. P38MAPK inhibits the Na^+/K^+ ATPase

p38MAPK was reported to affect the activity of the Na^+/K^+ ATPase, thus its involvement in the effect of Procyanidin B2 on the Na^+/K^+ ATPase was investigated. This was done by treating Caco-2 cells with exogenous PGE2 in the presence of SB 202190, an inhibitor of p38MAPK. SB 202190 alone induced a significant increase in the activity of the Na^+/K^+ ATPase, suggesting that p38MAPK exerts an inhibitory effect that is relieved in presence of the inhibitor SB 202190. When cells were treated with PGE2 and SB 202190 simultaneously, an additive stimulatory effect was observed (Fig.16).

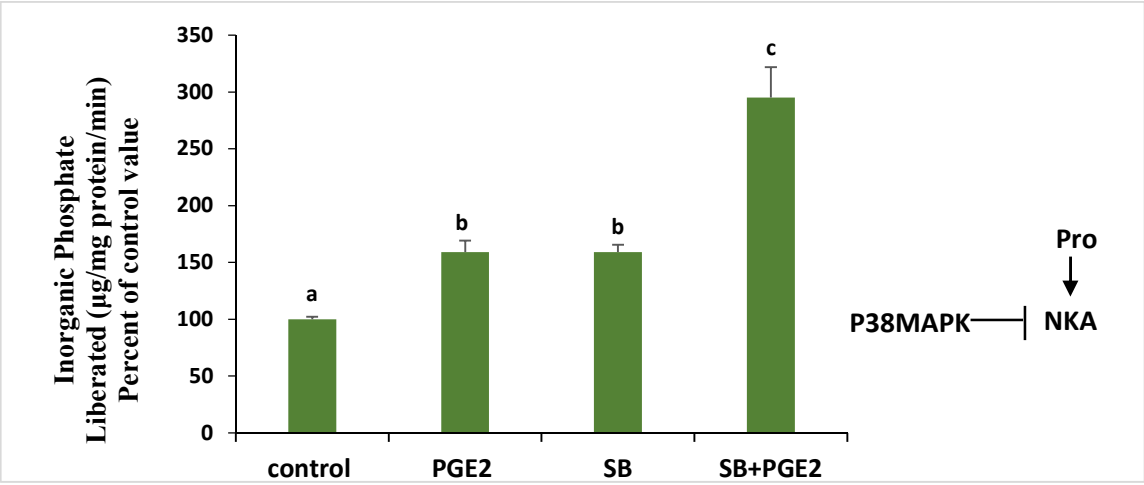

Fig.16. The Na^+/K^+ ATPase activity increased in presence of SB 202190 (50 µM), an inhibitor of p38MAPK and was increased further in the simultaneous presence of PGE2. The values are means ± SEM of 3 readings. The bars that show different letters are significantly different at $P<0.05$ as shown by ANOVA followed by Tukey test. SB= SB 202190, NKA= Na^+/K^+ ATPase, Pro= Procyanidin B2.

L. The effect of Procyanidin B2 on the expression of the Na^+/K^+ ATPase

To test whether the effect of Procyanidin B2 is limited to increasing the ATPase activity or it is also associated with an increase in its abundance, Caco-2 cells were treated with Procyanidin B2 (17µM, 2hrs) and changes in the protein expression of the Na^+/K^+ ATPase were tested by Western Blot analysis. Procyanidin B2 had no effect on the expression of the ATPase (Figs.17 and 18).

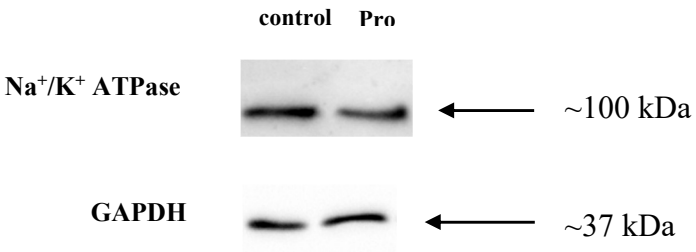

Fig.17. Procyanidin B2 has no effect on the expression of the Na^+/K^+ ATPase. GAPDH was used to test for equal loading. 40 µg of proteins were loaded in each well. Pro=Procyanidin B2. The blot is representative of an experiment repeated 3 times.

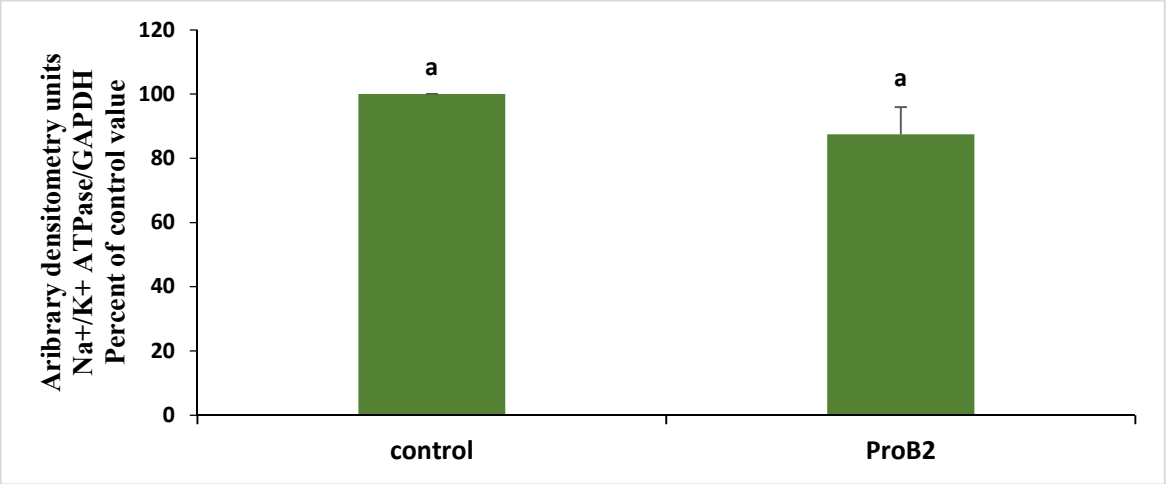

Fig.18. Procyanidin B2 has no effect on the expression of the Na^+/K^+ ATPase. Values are normalized to GAPDH using Image lab software. The values are means ± SEM of 4 readings. The bars that show the same letters are not significantly different at $P<0.05$ as shown by ANOVA followed by Tukey test. ProB2=Procyanidin B2.

CHAPTER V

DISCUSSION

Inflammatory Bowel Disease (IBD), which includes Crohn's Disease (CD) and Ulcerative Colitis (UC), is a colonic inflammatory disorder that is driven by several factors and characterized by an unusual immune response against the intestinal commensal flora (Wallace et al., 2014). The Na^+/K^+ ATPase (also known as the Na^+/K^+ pump) is an integral protein located in the basolateral membrane of all epithelial cells and responsible for the establishment and maintenance of the membrane electrochemical gradient. It mediates the transport of several ions across the membrane, mainly Na^+, K^+, Cl^-, and HCO^-_3, and thus controls the movement of water across the cell. The literature reports downregulation of the Na^+/K^+ ATPase in IBD (Silva and Soares-da-Silva, 2012; Surawicz, 2010).

On the other hand, Procyanidins were shown to have a protective role against the harmful effects of IBD. Procyanidins are phenolic compounds that are considered a subclass of flavonoids and are abundant in grapes, berries, and cocoa (Hammerstone et al., 2000; Robbins et al., 2012). The procyanidins in cocoa prevented the loss of barrier function in epithelial inflammation of the gut (Bitzer et al., 2015b), and those in apple exerted anti-inflammatory and immunomodulatory effects on epithelial cells in the intestine (Yoshioka et al., 2008). Since a change in the activity of the Na^+/K^+ ATPase is one of the characteristics of IBD, the involvement of procyanidins in this effect was questioned. This work was conducted to address this issue using Caco-2 cells as a model. An attempt was also done to explore the mediators present along the signaling pathway.

The results obtained showed a significant stimulatory effect of Procyanidin B2 on the Na^+/K^+ ATPase with no effect on it expression (Figs.17 and 18). A dose and time response study revealed maximal activation at a 17 μM concentration and a two-hour incubation period (Figs1 &2). This is in line with a study performed by Qi et al, which showed a similar stimulation of the cardiac Na^+/K^+ ATPase in hearts treated with epicatechin-3-gallate (ECG), one of the polyphenolic compounds of green tea, in the course of protection against Ischemia/Reperfusion Injury (Qi et al., 2019). Chandra et al. (2015) reported however inhibition of the thyroid Na^+/K^+ ATPase by peanut seed coats (PSC), that are known to be rich in phenolic and cyanogenic compounds. It is thus apparent that procyanidins and polyphenols modulate Na^+/K^+ ATPase activity in a tissue specific manner. A study performed by Li et al, showed that Larch bark procyanidins (LBPCs) induced a 1.86-fold increase in the activity of the Na^+/K^+ ATPase in *Staphylococcus aureus* after a 6 hour-treatment with 1 MIC (minimum inhibitory concentration) of LBPC (Li et al., 2017) suggesting that polyphenolic compounds might have an effect even on the Na^+/K^+ ATPase in prokaryotes. Despite the fact that procyanidins were extensively studied for their beneficial effects in different fields, yet their exact mode of action is still ambiguous. Whether they exert their effect by binding to membrane receptors or after crossing the membrane is still an unresolved issue. While some studies showed that a small percentage, around 1% of Procyanidin B2 and Procyanidin B5, could pass through the membrane of enterocytes (Spencer et al., 2001b), other studies reported that Procyanidin B2 cannot cross the cellular membrane (Li et al., 2019). No transporters for epicatechin in intestinal epithelial cells were reported in the literature, and the movement of procyanidin oligomers across the membrane was suggested to occur via permeation or diffusion

(Appeldoorn et al., 2009). Lately, it has been reported that certain procyanidins may interact with specific isoforms of the Human Bitter Taste Receptors (TAS2Rs) through specific protein-polyphenol interactions (Soares et al., 2018). Interestingly, certain isoforms of bitter taste receptors were shown to be expressed in mouse intestinal villus and crypts and in human colonic mucosa (Gu et al., 2015; Kaji et al., 2009), supporting the hypothesis that Procyanidin B2 may exert its effect through an interaction with TAS2Rs.

TAS2Rs are receptors coupled to the G-protein "gustducin". Activation of these receptors promotes the dissociation of the G-protein, and stimulates a signaling cascade that results in PKC activation (Avau et al., 2015; Kim et al., 2014; Yu et al., 2015; Yue et al., 2018). Thus, PKC was one of the potential mediators whose involvement was tested. PKC inhibition with Calphostin C abolished the stimulation induced by Procyanidin B2, and PKC stimulation with PMA induced a significant increase in the ATPase activity (Fig.9). These results indicate that PKC is involved in the signaling pathway and are in line with another study showing upregulation of PKC by grape seed procyanidins in the context of improving the proliferative activity of hepatic cells in mice (Liu et al., 2007).

Prostaglandin E2 (PGE2) plays a pivotal role in inflammation and inflammatory bowel disease (Montrose et al., 2015; Wallace, 2001). This is why its involvement in the effect of Procyanidin B2 on the Na^+/K^+ ATPase was investigated. The effect of Procyanidin B2 disappeared in presence of indomethacin, an inhibitor of COX enzymes, and a similar stimulatory effect was observed in presence of 100 nM exogenous PGE2 (Figs.3 and 4), confirming that PGE2 is a mediator in the signaling pathway. These results are consistent

with other studies showing upregulation of cyclooxygenase (COX) by grape seed procyanidins (Dasilva et al., 2017).

When Caco-2 cells were treated with PMA in presence of indomethacin, the previously observed stimulatory effect of PMA on the Na^+/K^+ ATPase disappeared (Fig.12), suggesting that PKC exerts its effect through PGE2 and is upstream the prostaglandin. This comes in line with previous work reporting a PKC-induced production of PGE2, mediated via NF-κB (Al Alam and Kreydiyyeh, 2016).

PGE2 is known to act by binding to four isotypes of EP receptors (EP_{1-4}) (Funk, 2001), thus the specific type of EP receptor involved was to be determined. Treating Caco-2 cells with procyanidin B2 in presence of SC-19220, PF-04418948, or L-798106, the respective blockers of EP1, EP2, and EP3, did not eliminate the effect of Procyanidin B2, but the presence of BGC-201531, an EP4 blocker (Figs.5,6,7,8) did. Therefore, PGE2 acts via EP4 in the signaling pathway induced by Procyanidin B2.

EP4 is linked to Gs, and its activation leads to the stimulation of adenylate cyclase which ultimately causes PKA activation (Dey et al., 2006b; Ichikawa et al., 2010a; Regan, 2003a). EP4, also activates PI3K/Akt (Fujino et al., 2003a). Since Procyanidin B2 was shown to act through EP4, the involvement of PKA and PI3K was checked.

Treating Caco-2 cells with RpcAMP, a PKA blocker, abolished the stimulatory effect of Procyanidin B2, while dbcAMP, a cell permeable cAMP analogue, showed a stimulatory effect (Fig.10). The results indicate thus an involvement of PKA in the signaling pathway. The stimulatory effect of PGE2 (100 nM), did not appear in presence of

RpcAMP (Fig.15), implying that PKA acts downstream of PGE2 and is activated by EP4 receptors. Activation of PKA by procyanidins was reported in the literature (Pinent et al., 2005). The cAMP-PKA pathway was shown to increase the activity of the Na^+/K^+ ATPase of the renal cortex in male Wistar rats (Bełtowski et al., 2003) and prevent colitis by reducing the expression of inflammatory mediators (Sun et al., 2017).

Since EP4 receptors activate PI3K, the role of the kinase was investigated. In presence of wortmannin, an inhibitor of PI3K, the stimulatory effect of Procyanidin B2 (Fig.11) was not observed, but unexpectedly, the effect of PGE2 still appeared (Fig.13), indicating that PI3K, similarly to PKC, lies upstream PGE2. Since PKC and PI3K were both shown to fall upstream PGE2, these two mediators were positioned with respect to each other. The effect of PMA still appeared in presence of wortmannin (Fig.14), indicating that PI3K is upstream PKC. PI3K-induced activation of PKC is a well-recognized process. The Gβγ subunit of gustducin activates PLC-β which generates DAG needed for the activation of cPKCs and nPKCs. The kinases associate with membrane bound DAG through C1 domains. The 3-phosphorylated phosphatidylinositol products of PI3K such as PtdIns(3,4,5)P3 provide docking sites for phosphoinositide-dependent kinase-1 PDK-1 which can now phosphorylate and activate PKC (Newton, 2010; Parker and Parkinson, 2001; Taylor and Kornev, 2011, Cenni et al., 2002, Facchinetti et al., 2008), after they have been both recruited to the membrane

In fact, some studies showed that PKC is an effector downstream PI3K, where epigallocatechin (EGC) induced the phosphorylation of PI3K and the downstream PKC λ/ξ

in skeletal muscles (Ueda-Wakagi et al., 2018). Another study also reported that PKC falls downstream PI3K in the pathway mediating the enhanced collagen gel contraction by platelet-derived growth factor-BB (Reyhani et al., 2017).

The literature reports also a role of p38MAPK in inducing PGE2 signaling (El Moussawi et al., 2018) as well as in inflammation and inflammatory bowel disease, where the combination of the inflammatory cytokines TNFα and IL-17, caused an upregulation in the expression of NOXO1, through an activation of p38MAPK and JNK1/2 (Makhezer et al., 2019). To investigate the involvement of p38MAPK, Caco-2 cells were treated with PGE2 in presence and absence of SB-202190, an inhibitor of p38 MAPK. The inhibitor alone, induced a stimulatory effect similar to that observed with exogenous PGE2 (100 nM). The simultaneous treatment with PGE2 (100 nM) and SB 202190 displayed an additive stimulatory effect on the pump (Fig.16). These results imply that the Na^+/K^+ ATPase activity is reduced by the basal activity of p38MAPK, and enhanced when the kinase is inhibited. Procyanidin B2 and PGE2 activate the Na^+/K^+ ATPase through a p38 MAPK independent pathway. Had p38MAPK been along the signaling pathway then no additive effect would have been observed.

It can be concluded that Procyanidin B2 (17 μM, 2 hrs) increases the activity of the Na^+/K^+ ATPase but does not affect its expression. This increase is mediated through PI3K which activates PKC. The latter induces the production of PGE2 which activates PKA via EP4 receptors. PKA then enhances directly or indirectly the activity of the Na^+/K^+ ATPase. Additional work must be done to verify the hypothesis that procyanidin B2 acts vis TAS2Rs, and explain how these receptors are linked to PI3K.

P38MAPK modulates the ATPase activity but is not along the signaling pathway of procyanidin B2.

The results suggest that Procyanidins could alleviate some of the symptoms of IBD by enhancing the activity of the Na^+/K^+ ATPase in the colon, increasing water absorption and reducing diarrhea. The figure below represents the signaling pathway

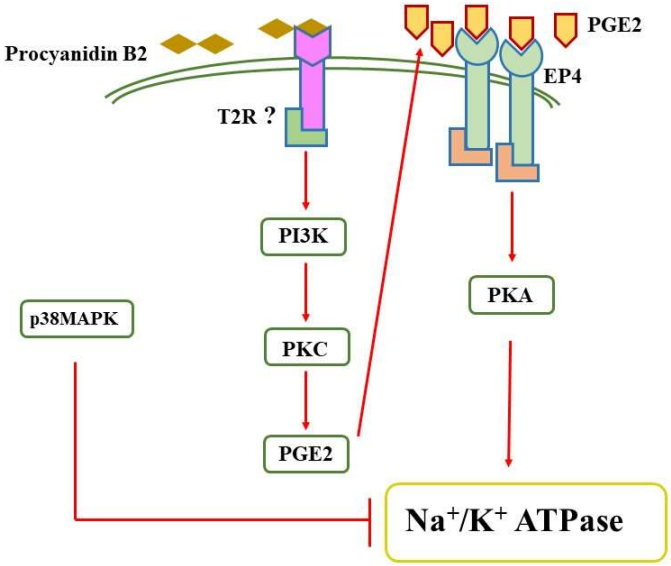

Fig.19. Figure showing the proposed signaling pathway mediated by Procyanidin B2, leading to the activation of the Na^+/K^+ ATPase in Caco-2 cells.

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