

## RESEARCH ARTICLE

# Adverse effect of FTY720P on colonic Na<sup>+</sup>/K<sup>+</sup> ATPase is mediated via ERK, p38MAPK, PKC, and PI3K

Reem Rida<sup>1</sup> | Rawad Hodeify<sup>2</sup> | Sawsan Kreydiyyeh<sup>1</sup> 

<sup>1</sup>Department of Biology, Faculty of Arts and Sciences, American University of Beirut, Beirut, Lebanon

<sup>2</sup>Department of Biotechnology, School of Arts and Sciences, American University of Ras Al Khaimah, Ras Al Khaimah, United Arab Emirates

**Correspondence**

Sawsan Kreydiyyeh, Department of Biology Faculty of Arts and Sciences American University of Beirut, Beirut, Lebanon.  
Email: [sawkreyd@aub.edu.lb](mailto:sawkreyd@aub.edu.lb)

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**Abstract**

FTY720P, an analogue of sphingosine 1-phosphate, has emerged lately as a potential causative agent of inflammatory bowel disease, in which electrolytes movements driven by the sodium gradient established by the Na<sup>+</sup>/K<sup>+</sup> ATPase are altered. We showed previously in Caco-2 cells, a 50% FTY720P-induced decrease in the ATPase activity, mediated via S1PR2 and PGE2. This work aims at delineating the mechanism underlying PGE2 release and at investigating if the ATPase inhibition is due to changes in its abundance. The activity of the ATPase and the localization of a GFP-tagged Na<sup>+</sup>/K<sup>+</sup>-ATPase  $\alpha_1$ -subunit were assessed in cells treated with 7.5 nM FTY720P. The involvement of ERK, p38 MAPK, PKC, and PI3K was studied in cells treated with 7.5 nM FTY720P or 1 nM PGE2 in presence of their inhibitors, or by determining changes in the protein expression of their activated phosphorylated forms. Imaging data showed ~30% reduction in the GFP-tagged Na<sup>+</sup>/K<sup>+</sup> ATPase at the plasma membrane. Both FTY720P and PGE2 showed, respectively, 50% and 60% reduction in ATPase activity that disappeared when p38 MAPK, PKC, and PI3K were inhibited individually but not with ERK inhibition. The effect of FTY720P was imitated by PMA, an activator of PKC. Western blotting revealed inhibition of ERK by FTY720P. It was concluded that FTY720P, through activation of S1PR2, downregulates the Na<sup>+</sup>/K<sup>+</sup> ATPase by inhibiting ERK, which in turn activates p38 MAPK leading to the sequential activation of PKC and PI3K, PGE2 release, and a decrease in the Na<sup>+</sup>/K<sup>+</sup> ATPase activity and membrane abundance.

**KEYWORDS**

Caco-2, ERK, FTY720P, Na<sup>+</sup>/K<sup>+</sup> ATPase, p38MAPK, PGE2, PI3K, PKC

## 1 | INTRODUCTION

FTY720P or fingolimod phosphate, an active immune modulator (Yu et al., 2022), has been recognized as a structural analogue of sphingosine 1-phosphate (S1P). It has been approved by the Food and Drug Administration for the treatment of multiple sclerosis and showed

promising results in the treatment of cancer (White et al., 2016). Bradycardia, increased blood pressure, and higher sensitivity to infections (Fragoso, 2017; Murphy et al., 2012) were recognized as some of its side effects. Whether the drug exerts any adverse effects on colonic activities and the Na<sup>+</sup>/K<sup>+</sup> ATPase is an area that has not been explored before.

**Abbreviations:** cAMP, cyclic adenosine monophosphate; COX, cyclooxygenase; dbcAMP, 2'-O-dibutyryl adenosine 3',5'-cyclic monophosphate sodium salt; ERK, extracellular signal-regulated kinase; FTY720, fingolimod; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; IBD, inflammatory bowel disease; Na<sup>+</sup>/K<sup>+</sup> ATPase, sodium potassium ATPase; NO, nitric oxide; p38 MAPK, p38 mitogen-activated protein kinase; PGE2, prostaglandin E2; PIP2, phosphatidylinositol 4,5-bisphosphate; PKA, protein kinase A; PKC, protein kinase C; PI3K, phosphoinositide 3-kinase; PLC, phospholipase C; PMA, phorbol 12-myristate 13-acetate; RpcAMP, adenosine-3',5'-cyclic monophosphorothioate Rp-isomer triethylammonium salt; S1P, sphingosine-1-phosphate; S1PR, sphingosine-1-phosphate receptor.

The enteric  $\text{Na}^+/\text{K}^+$  ATPase or  $\text{Na}^+/\text{K}^+$  pump plays a pivotal role in the absorption of electrolytes and water and in driving various sodium dependent secondary active transport processes. Any alteration in its activity may lead to diarrhea or constipation. A decrease in the pump's activity (Magalhães et al., 2016) ascribed to a reduced protein expression of the alpha subunit (Greig & Sandle, 2000) was observed in inflammatory bowel disease (IBD) patients coupled to higher levels of S1P (Murphy et al., 2012), suggesting a role of S1P, and probably of its analogue FTY720P, in modulating the ATPase activity. This hypothesis was confirmed in a previous work (Rida & Kreydiyyeh, 2018) conducted on Caco-2 cells, which demonstrated a significant decrease in the activity of the  $\text{Na}^+/\text{K}^+$  pump by FTY 720P, that was mediated via sphingosine one phosphate receptor 2 (S1PR2) and prostaglandin E2 (PGE2). This lower activity may result from a reduced specific activity of the ATPase or from a decrease in the abundance of the ATPase units in the membrane. Pump units are known to traffic between the membrane and an intracellular pool (Barlet-Bas et al., 1990) in order to serve the needs of the cell and secure proper functioning and homeostasis. A translocation of the ATPase to the plasma membrane was observed in HepG2 cells treated with PGE2 (Hodeify et al., 2021). Internalization of the pump was reported to be triggered by some kinases (Beron et al., 1997) and may or may not be accompanied with its phosphorylation, suggesting that the ATPase trafficking may result from its phosphorylation or the phosphorylation of intermediate molecules present along the signaling pathway. Whether the FTY720P-induced decrease in the ATPase activity results from a decrease in the number of pump molecules present in the membrane is a question that has not been addressed in our previous work and constitutes one of the aims of the current study. Another aim of this work is the elucidation of the mechanism underlying the induced PGE2 release and eventually the downregulation of the  $\text{Na}^+/\text{K}^+$  ATPase. Because the ATPase plays a crucial role in electrolytes and water movements in the colon, determining the signaling cascade activated by the FTY720P would help, by modulating the activity of the intermediate signaling molecules, in treating the side effects of the drug on colonic transport processes. Since FTY720P induces PGE2 release and since the prostaglandin is involved in various colonic activities like modulation of colonic motility (Karaki & Tanaka, 2021) and colorectal cancer (Eberhart et al., 1994), delineating the signaling pathway leading to its release may allow for an intervention that would block its production during FTY720P treatments and consequently the circumvention of any undesirable effect induced by the drug.

## 2 | MATERIALS AND METHODS

### 2.1 | Materials

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

Anti-ERK 1/2 rabbit polyclonal antibody was from Promega, WI, USA, while anti-p-ERK 1/2 rabbit polyclonal antibody was from Cell Signaling, MA, USA.

Phorbol-12-myrsitate-13-acetate (PMA), SB 202190, Calphostin C, wortmannin, and PD98059 were obtained from Calbiochem, San Diego, USA.

FTY720-P, the primary antibodies anti p38 MAPK, anti p-p38MAPK, anti-GAPDH, and HRP conjugated secondary antibody were provided from Santa Cruz Biotechnology, CA, USA.

Biorad protein assay reagent, nitrocellulose membranes, western blotting luminol, and peroxidase (Clarity TM Western ECL Substrate) reagent were obtained from Biorad, California, USA.

Prostaglandin E2 (PGE2), indomethacin, ouabain, Dulbecco's minimal essential medium (DMEM) with 4500 mg/L glucose and pyridoxine HCL, Trypsin-EDTA, penicillin/streptomycin, fetal bovine serum (FBS), 10× phosphate buffered saline (PBS) without magnesium and calcium, adenosine 5'-triphosphate disodium salt (ATP), and all other chemicals were procured from Sigma, Chemical Co, St Louis, Missouri, USA.

GFP-tagged Na-K-ATPase plasmid was a generous gift from Sznajder Laboratory, Northwestern University, and mCherry-tagged membrane plasmid was a gift from Catherine Berlot (Addgene plasmid # 55779).

## 2.2 | Methods

### 2.2.1 | Culture and treatment of Caco2 cells

Caco2 cells at passages 32–50 were cultured in a humidified incubator (95%  $\text{O}_2$ , 5%  $\text{CO}_2$ ) at 37°C in DMEM supplemented with 1% penicillin (100  $\mu\text{g}/\text{ml}$ ), streptomycin (100  $\mu\text{g}/\text{ml}$ ), and 10% FBS in six-well plates at a density of 1,000,000 cells per well. The cells were treated at 85–90% confluence after an overnight starvation.

In all treatment, an equal volume of the vehicle was always added to the control.

### 2.2.2 | Treatment of cells with FTY720P

Caco-2 cells were treated, after an overnight starvation, with 7.5 nM FTY720-P for 15 min, or with PGE2 (1 nM, 15 min). At the end of the incubation period, the cells were collected, homogenized, and spun at 4°C for 30 min at 2000g. Proteins in the supernatant were quantified according to the Bradford method and used to assay for the  $\text{Na}^+/\text{K}^+$  ATPase activity or for western blotting.

### 2.2.3 | Testing the involvement of PKC, PI3K, p38MAPK, and ERK

Protein kinase C (PKC) was reported to be involved in the  $\text{Na}^+/\text{K}^+$  ATPase trafficking. On the other hand, S1PR2 receptors are coupled to Gq and Gi/o, which both may activate phospholipase C (PLC) and consequently PKC. Hence, PKC was considered as a potential

mediator and its involvement in the effect of FTY720P was investigated by treating the cells with calphostin C (50 nM), a PKC inhibitor, 1 h before FTY720P, or by treating the cells for 15 min directly with 100 nM phorbol 12-myristate 13-acetate (PMA), a PKC activator.

The literature reports also phosphoinositide 3-kinase (PI3K), AKT kinase (Ren et al., 2017), p38 mitogen activated protein kinase (MAPK), and extracellular signal-regulated kinase (ERK) (Greenspon et al., 2009; Kwong et al., 2015) as downstream effectors of S1PR2. On the other, the  $\text{Na}^+/\text{K}^+$  ATPase is recognized as a target of all of these kinases (Peruchetti et al., 2011). Consequently, the involvement of these mediators was investigated. To know if p38MAPK and ERK are along the signaling pathway, cells were pre-treated, 1 h before FTY720P, with their respective inhibitors wortmannin (100 nM), SB202190 (50  $\mu\text{M}$ ), and PD98059 (50  $\mu\text{M}$ ).

## 2.2.4 | Locating the various mediators with respect to each other in the signaling pathway

To know if PKC is upstream or downstream PGE2, Caco2 cells were treated with PMA (100 nM, 15 min), after a pre-treatment with indomethacin (100  $\mu\text{M}$ , 1 h), an inhibitor of PGE2 synthesis, or with calphostin, a PKC inhibitor, in presence of PGE2. Its location on the pathway relative to p38MAPK was studied by treating the cells with PMA (100 nM, 15 min) following a pre-treatment with SB203580 (50  $\mu\text{M}$ ), for 1 h.

The position of PI3K, p38MAPK, and ERK relative to PGE2 was investigated by treating the cells with PGE2 (1 nM, 15 min) in presence of their respective inhibitor wortmannin (100 nM), SB203580 (50  $\mu\text{M}$ ), or PD98059 (50  $\mu\text{M}$ ), added to the cells 1 h prior to the prostaglandin.

To investigate further the position of p38MAPK with respect to PKC and ERK, changes in the protein expression of p-p38MAPK were determined by western blot analysis when ERK and PKC were inhibited.

## 2.2.5 | $\text{Na}^+/\text{K}^+$ ATPase activity assay

The activity of the  $\text{Na}^+/\text{K}^+$  ATPase was assayed by measuring the amount of inorganic phosphate liberated in presence and absence of ouabain, a specific inhibitor of the ATPase, as described by Esmann (1988). Briefly, the protein concentration of cell homogenates was adjusted to 0.5  $\mu\text{g}/\mu\text{l}$  with histidine buffer (pH 7.4, 150 mM). Samples were then incubated for 15 min at room temperature with 1% saponin added at a ratio of 1:4, in presence of phosphatase inhibitors (2.7 mM pyrophosphate, 2.7 mM glycerophosphate). Aliquots were then taken from each sample and incubated in histidine buffer containing NaCl (121.5 mM), KCl (19.6 mM),  $\text{MgCl}_2$  (3.92 mM), adenosine triphosphate (2.94 mM), and in presence or absence of ouabain (1.47 mM). When ouabain was absent, it was replaced with water. The reaction was stopped by addition of 50% trichloroacetic acid at a ratio of 1:10 (v/v), and the samples were spun at 3000g for 5 min. The amount of inorganic phosphate liberated in the supernatant was

measured colorimetrically at 750 nm according to the method of Taussky and Shorr (1953). The results are reported as percentage of control values.

## 2.2.6 | Western blot analysis

Forty micrograms of cell homogenate proteins were run on 10% SDS polyacrylamide and then transferred to nitrocellulose membranes. The membranes were blocked and then incubated overnight at 4°C with anti-AKT, anti-p38MAPK, and anti-p-AKT and anti-p-p38MAPK primary antibodies, followed with an incubation with HRP conjugated secondary antibodies for 1 h at room temperature. The signal was detected by chemiluminescence using Clarity Western ECL Substrate. The signals were digitized and the data analyzed using ChemiDoc™ MP imaging system.

## 2.2.7 | Cell transfection and imaging

Caco2 cells seeded on poly-D-lysine coated glass-bottomed dishes (MatTek) were co-transfected with GFP-tagged  $\text{Na}^+/\text{K}^+$  ATPase plasmid (a generous gift from Sznajder Laboratory, Northwestern University) and mCherry-tagged membrane plasmid (a gift from Catherine Berlot (Addgene plasmid # 55779) 1 day after seeding. Forty-eight hours after transfection, cells were treated with FTY720P (7.5 nM for 15 min) followed by imaging using confocal microscope Zeiss LSM710. For each cell, fluorescence ratio of GFP/mCherry was obtained using Image J to determine Na-K-ATPase expression on plasma membrane in control compared with treated cells.

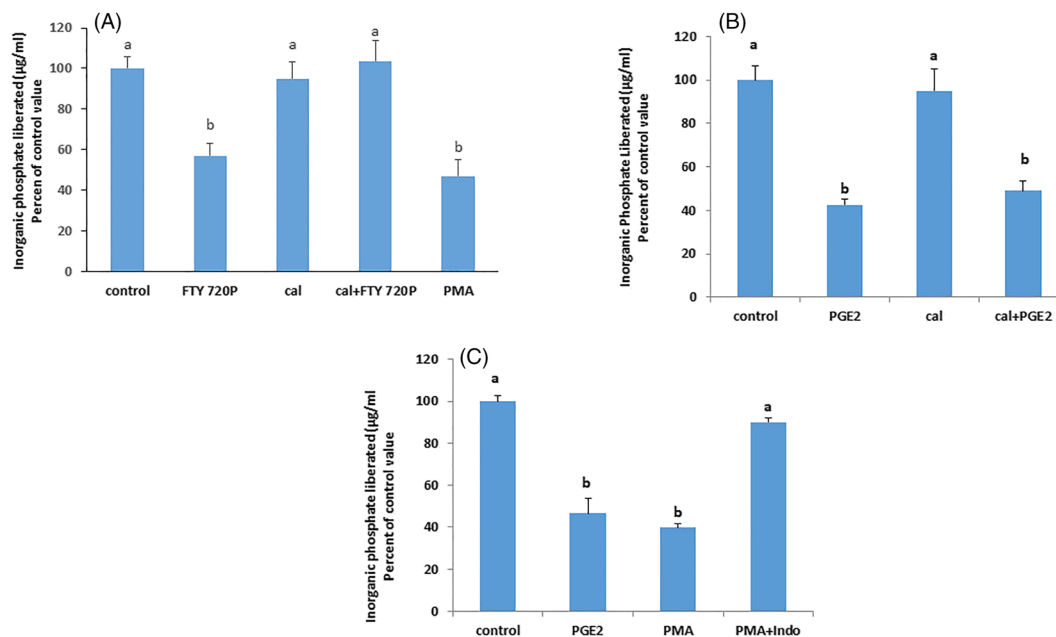
## 2.2.8 | Statistical analysis

The results are reported as mean  $\pm$  SEM and tested for statistical significance using a one-way analysis of variance followed by a Tukey–Kramer multiple comparison test using GraphPad InStat 3.

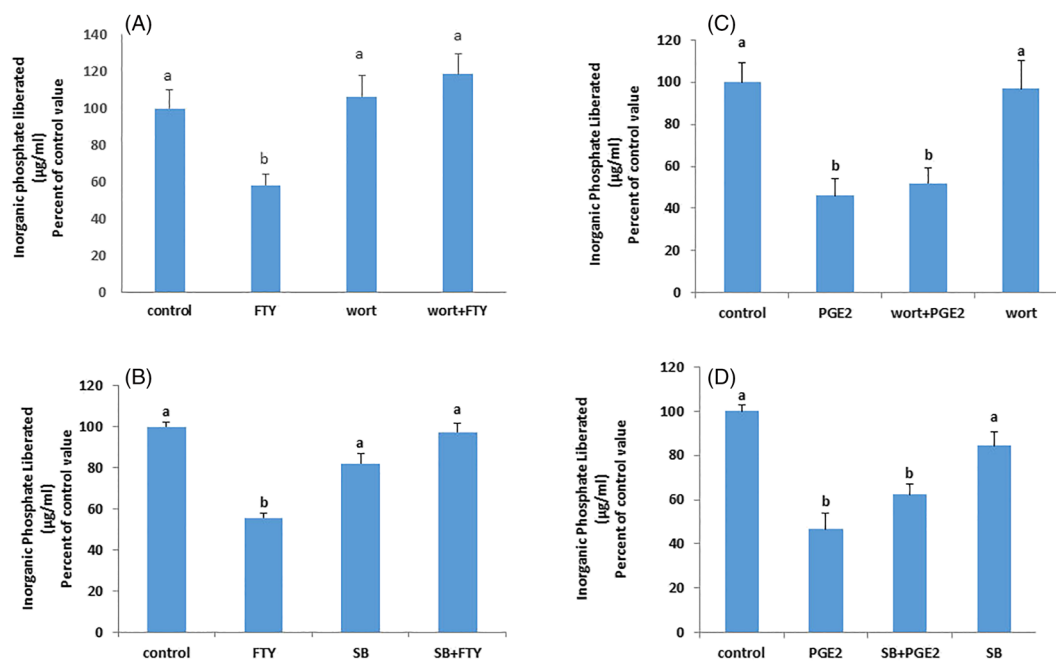
# 3 | RESULTS

## 3.1 | PKC, PI3K, p-38MAPK, and ERK are involved in the signaling pathway

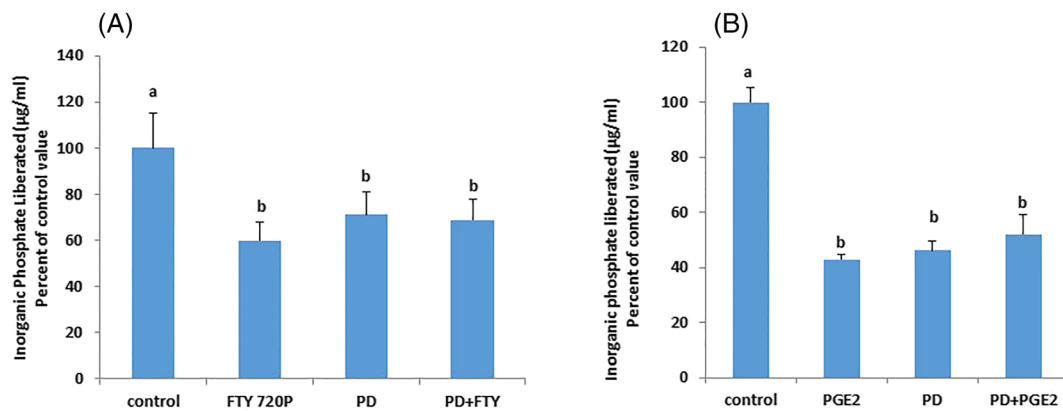
Treating Caco-2 cells with 7.5 nM FTY720P led to 50% reduction in the activity of the  $\text{Na}^+/\text{K}^+$  ATPase. Similarly, 1 nM PGE2 led to 60% reduction in the pump's activity. The effect of FTY720P (Figure 1A) but not that of PGE2 (Figure 1B) was abrogated in presence of calphostin, an inhibitor of PKC, and was imitated by PMA (Figure 1A), a PKC activator. The inhibition observed with PMA did not appear however when PGE2 synthesis was blocked with indomethacin (Figure 1C), indicating the presence of PKC along the signaling pathway upstream of PGE2.



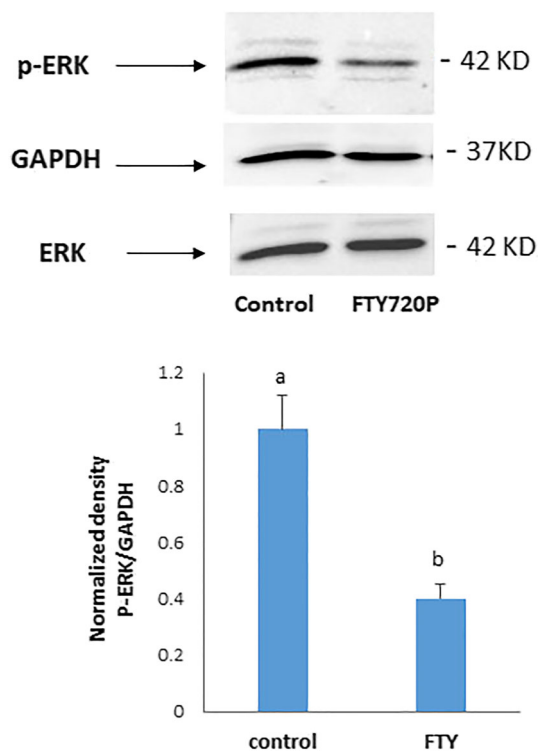
**FIGURE 1** PKC is a mediator of FTY720P's action. (A) Calphostin C (50 nM) added to the cells 1 h before FTY720P (7.5 nM, 15 min) abolished the effect of FTY720P (7.5 nM, 15 min) on the  $\text{Na}^+/\text{K}^+$  ATPase while PMA (100 nM, 15 min) exerted a similar inhibitory effect. (B) Effect of PGE2 (1 nM, 15 min) on the  $\text{Na}^+/\text{K}^+$  ATPase activity in presence of calphostin C (50 nM) added 1 h earlier. (C) The effect of PMA (100 nM, 15 min) disappeared when the cells were pre-treated with indomethacin (100 µM, 1 h). Values are means  $\pm$  SEM of three observations. Bars not sharing a common letter are significantly different from each other at  $p < 0.01$ . [Colour figure can be viewed at [wileyonlinelibrary.com](https://onlinelibrary.wiley.com/doi/10.1002/jat.4375)]



**FIGURE 2** PI3K and p38 MAPK are along the signaling pathway. Effect of FTY720P on the  $\text{Na}^+/\text{K}^+$  ATPase activity after a pre-treatment for 1 h with (A) wortmannin (100 nM) or (B) SB202190 (50 µM). Effect of PGE2 on the  $\text{Na}^+/\text{K}^+$  ATPase activity after a pre-treatment for 1 h with (C) wortmannin (100 nM) or (D) SB202190 (50 µM). Values are means  $\pm$  SEM of three observations. Bars not sharing a common letter are significantly different from each other at  $p < 0.01$ . [Colour figure can be viewed at [wileyonlinelibrary.com](https://onlinelibrary.wiley.com/doi/10.1002/jat.4375)]



**FIGURE 3** Effect of FTY720P and PGE2 on the activity of the  $\text{Na}^+/\text{K}^+$  ATPase after a 1-h pre-treatment with PD98059 (50  $\mu\text{M}$ ). Values are means  $\pm$  SEM of at least three observations. Bars not sharing a common letter are considered significantly different from each other at  $p < 0.01$ . [Colour figure can be viewed at [wileyonlinelibrary.com](http://wileyonlinelibrary.com)]



**FIGURE 4** Effect of FTY720P on the protein expression of (A) p-ERK and (B) total ERK. Values are normalized to GAPDH using Image lab software. The blot is representative of an experiment repeated three times. Values are normalized to GAPDH. Bars not sharing a common letter are considered significantly different from each other at  $p < 0.01$ . [Colour figure can be viewed at [wileyonlinelibrary.com](http://wileyonlinelibrary.com)]

Inhibiting PI3K and p38MAPK with, respectively, wortmannin and SB203580 abolished the effect of FTY720P (Figure 2A,B), but not that of PGE2 (Figure 2C,D) suggesting its presence downstream the two kinases.

In presence of PD98059, an inhibitor of ERK, FTY720P (Figure 3A), and PGE2 (Figure 3B) were still able to exert their inhibitory effect on the pump. The inhibitor alone induced a 35–50% decrease in the activity of the ATPase. Western blot analysis revealed a 60% decrease in the protein expression of p-ERK by FTY720P (Figure 4).

### 3.2 | PKC is downstream p38MAPK and upstream PI3K in FTY720P mediated ATPase inhibition

PKC activation with PMA exerted an inhibitory effect on the ATPase that was maintained in presence of SB 203580 (Figure 5A), an inhibitor of p38MAPK, but disappeared in presence of wortmannin (Figure 5B), an inhibitor of PI3K,

### 3.3 | P38 is downstream ERK and upstream PKC

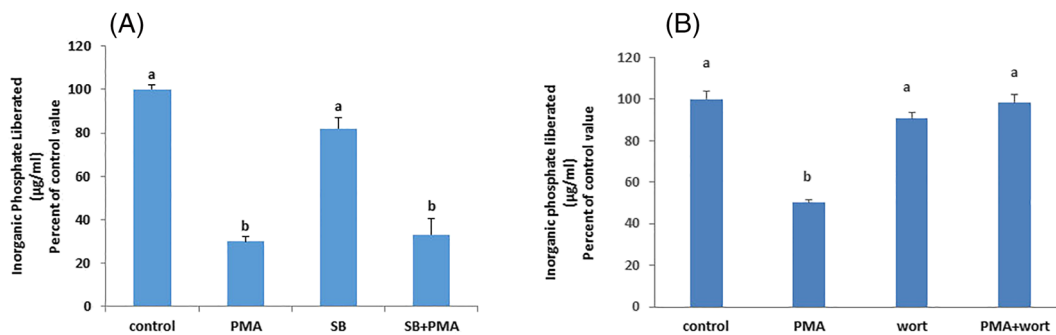
The FTY720P-induced increase in protein expression of p-p38 MAPK (around three folds) increased further when ERK was inhibited, but was not affected by PKC inhibition (Figure 6).

### 3.4 | $\text{Na}^+/\text{K}^+$ ATPase abundance in the plasma membrane in Caco-2 cells treated with FTY720P

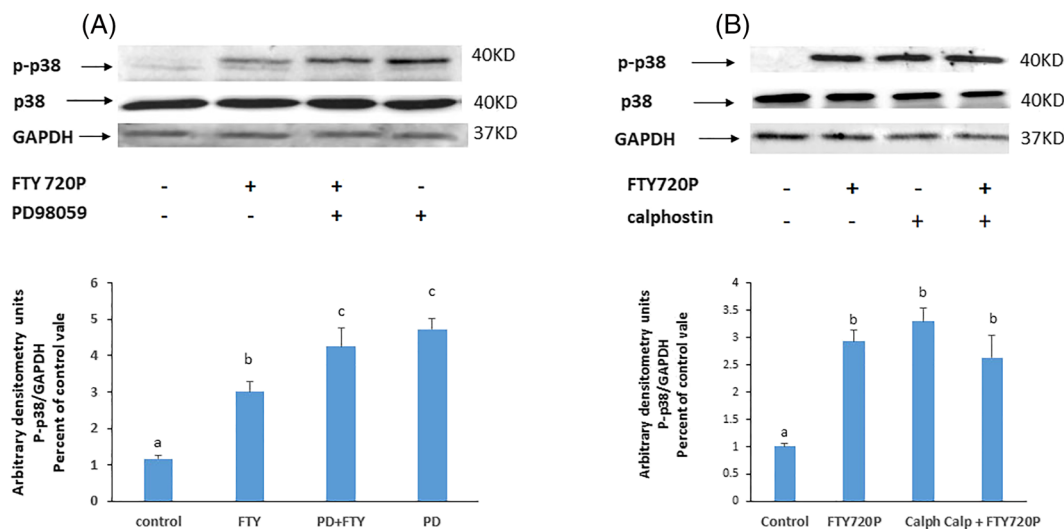
To study the effect of FTY720P on the abundance of  $\text{Na}^+/\text{K}^+$  ATPase in the plasma membrane of Caco-2 cells, GFP-tagged  $\text{Na}^+/\text{K}^+$  ATPase  $\alpha 1$  in the presence of a mCherry-membrane marker was assessed using fluorescence microscopy. Relative quantification of GFP/mCherry showed  $\sim 30\%$  decrease in FTY720P-treated cells as compared to control cells (Figure 7).

## 4 | DISCUSSION

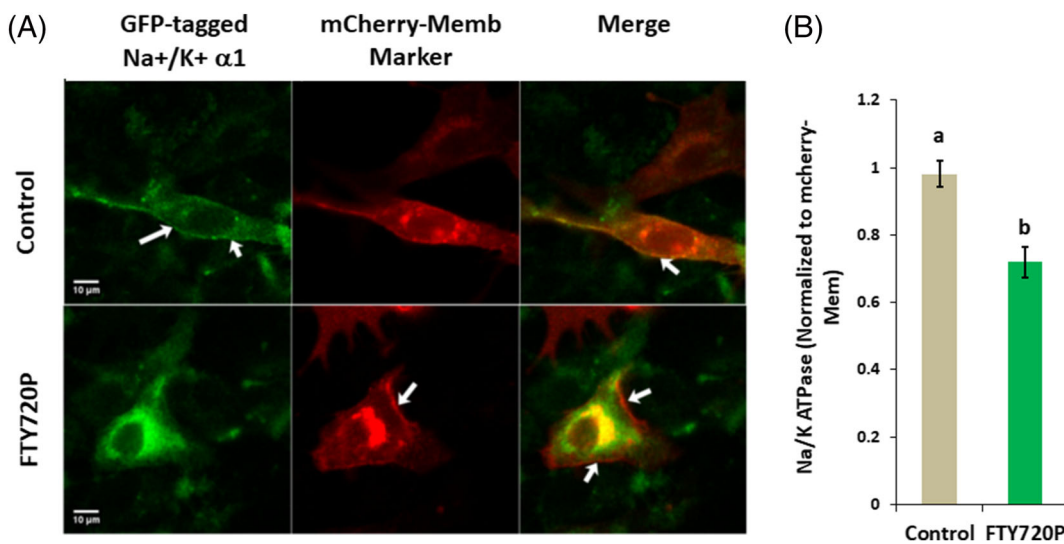
S1P, through S1PR2, plays an important role in maintaining the integrity of the intestinal epithelial cell barrier (Chen et al., 2017). By modulating the activity of various intestinal transporters, S1PR2 also regulates electrolytes and water movements. The  $\text{Cl}^-/\text{HCO}_3^-$  exchanger SLC26A3 is one of these transporters whose protein expression was found to be increased upon activation of S1PR2. The exchanger, through its coupled activity with the  $\text{Na}^+/\text{H}^+$  antiporter is responsible for the electroneutral NaCl absorption in the ileum and



**FIGURE 5** Effect of PMA in presence of (A) SB202190 (50 µM) or (B) wortmannin (100 nM) added 1 h earlier. Values are means ± SEM of at least three observations. Bars not sharing a common letter are considered significantly different from each other at  $p < 0.01$ .



**FIGURE 6** Effect of FTY720P on the protein expression of p-p38MAPK in cells pre-treated with (A) PD98059 (50 µM) or (B) calphostin C (50 nM) for 1 h. The blots are representative of an experiment repeated three times. Values are normalized to total p38MAPK. Bars not sharing a common letter are considered significantly different from each other at  $p < 0.01$ .



**FIGURE 7** FTY720P reduced the abundance of Na<sup>+</sup>/K<sup>+</sup> ATPase molecules in the plasma membrane. (A) Fluorescent images from Caco-2 cells co-expressing GFP-tagged Na<sup>+</sup>/K<sup>+</sup> α1 subunits and mCherry-membrane marker treated with FTY720P as compared with the control. (B) Bars not sharing a common letter are considered significantly different from each other at  $p < 0.01$ .

colon (Malakooti et al., 2011). The activity of the  $\text{Na}^+/\text{H}^+$  exchanger is driven by the sodium gradient established by the  $\text{Na}^+/\text{K}^+$  pump. In a previous study, we demonstrated a decrease in the activity of the ATPase upon S1PR2 activation that was mediated via PGE2. This work is an attempt to delineate the signaling cascade between FTY720P and PGE2, by studying the involvement of some mediators known to be modulated by S1PR2. In addition, changes in the abundance of the  $\text{Na}^+/\text{K}^+$  ATPase molecules present in the membrane was investigated.

The  $\text{Na}^+/\text{K}^+$  ATPase is known to be regulated by phosphorylation/dephosphorylation processes (Bertorello et al., 1991; Mohan et al., 2019) in response to hormones, neurotransmitters (Aperia et al., 1992), cytokines (Al-Sadi & Kreydiyyeh, 2003; Markossian & Kreydiyyeh, 2005), and inflammatory mediators (Nepal et al., 2021). The effect of phosphorylation on the ATPase, even by the same kinase, is tissue specific, varies from stimulation to inhibition (Satoh et al., 1992; Tria et al., 1974; Vasilets et al., 1990), and may or may not trigger the translocation of the ATPase units between intracellular pools and the plasma membrane (Kristensen et al., 2003; Lecuona et al., 2009).

ERK, p38MAPK, and PI3K were considered as potential mediators of FTY720P's action, since knockdown of S1PR2 in murine bone marrow-derived monocytes and macrophages suppressed the expression of p-PI3K, p-ERK, and p-p38, induced by the oral pathogen *A. actinomycetemcomitans* (Yu, 2016), suggesting that the effect of S1PR2 is mediated through these kinases. Consequently, they may be also along the signaling pathway activated by FTY720P. Hence, investigating their involvement was deemed necessary. S1PR2s are coupled to Gq, Gi, and G12/13. Gq activates phospholipase C leading to an increase in the level of diacylglycerol and calcium and activation of PKC (Adada et al., 2013). PKC was reported to modulate the  $\text{Na}^+/\text{K}^+$  ATPase activity either directly by phosphorylation (Feschenko et al., 2000) of residues at the N terminus (Beguín et al., 1994), or indirectly by phosphorylating and activating other intermediate molecules (Mohan et al., 2021) that would eventually alter the ATPase activity or membrane abundance. Hence, PKC may also be a possible mediator of FTY720P's action.

#### 4.1 | P38MAPK and ERK are along the pathway and ERK is upstream

The reduced ATPase activity observed with FTY720P and PGE2 still appeared when ERK was inhibited with PD98059, implying that either ERK is not involved, or if it is, it is inhibited by the sphingolipid and the prostaglandin. The status of ERK was clarified by western blot analysis, which demonstrated a reduction in its protein expression as compared with the control. This ERK inhibition may be mediated via the S1PR2-coupled  $\text{G}\alpha_{12}/13$  which was shown to reduce the activity of ERK1/2, (Voyno-Yasenetskaya et al., 1996) through a proposed mechanism involving activation of the ser/thr protein phosphatase PP5 by  $\text{G}\alpha_{12}/13$ . PP5 then inactivates Raf-1 by dephosphorylation at

Ser 333 (von Kriegsheim et al., 2006) leading to MEK and ERK inhibition.

Inhibition of p38MAPK abrogated the effect of FTY720P on the pump but not that of PGE2, indicating that the kinase is along the signaling pathway but upstream PGE2. Western blot analysis confirmed further the stimulatory effect of FTY720P on p38MAPK. An increase in the expression of p-p38MAPK was observed also in presence of PD98059 or in the simultaneous presence of PD98059 and FTY720P, indicating that an inhibition of ERK exerts a stimulatory effect on p38MAPK and is in line with the findings of Berra et al. (1998) who demonstrated clearly that the mere inhibition of ERK in Hela cells led to a significant stimulation of p38 MAPK which correlated with the antagonistic effect of the two kinases on apoptosis, with ERK inducing cell survival and p38 MAPK inducing cell death. Similarly, Xia et al. (1995) found also that apoptosis in PC12 cells is the result of the concurrent activation of p38 and inhibition of ERK.

#### 4.2 | PKC is involved

PKC but not PGE2 acts via PKC, since the inhibitory effect of FTY720P but not that of PGE2 was completely abolished by calphostin, and mimicked by PMA. A similar effect of phorbol esters (PMA) on the  $\text{Na}^+/\text{K}^+$  ATPase has been observed before (Bełtowski et al., 2004). The inhibitory effect of PMA did not appear in presence of the COX inhibitor, indomethacin, indicating that PKC is upstream PGE2. The literature reports such a role of PKC in PGE2 production (Ohama et al., 2008; Yang et al., 2020).

#### 4.3 | PI3K is a mediator in the FTY720P signaling pathway, and is located upstream of PGE2

Several studies revealed that S1PR2 acts via PI3K/Akt (Beckham et al., 2013; Yin et al., 2018). In this work also, activation of S1PR2 by FTY720P led to PI3K activation since the inhibitory effect of FTY720P did not appear in presence of wortmannin. Yu (2016) showed similarly that knockdown of S1PR2 in murine bone marrow-derived monocytes and macrophages treated with the oral pathogen *Aggregatibacter* resulted in a lower activity of PI3K as compared with the control.

PI3K can have a direct or an indirect effect on the  $\text{Na}^+/\text{K}^+$  pump. Dopamine, which acts via GPCRs, was shown to induce endocytosis of the  $\text{Na}^+/\text{K}^+$  ATPase through PI3K- $\text{I}_A$ , which binds to a proline rich sequence in the alpha subunit of the ATPase that becomes accessible only upon phosphorylation of a serine residue by PKC (Yudowski et al., 2000). Inhibition of myocardial  $\text{Na}^+/\text{K}^+$  ATPase during endotoxemia does not occur via a direct phosphorylation by PI3K but via activation of PI3K/Rac1/NADPH oxidase (Zhang et al., 2012).

The inhibitory effect of PGE2 appeared unchanged in presence of wortmannin implying that PI3K is upstream PGE2. These findings are in line with those of Hsieh et al. (2006) who showed in vascular

smooth muscle cells, a S1P-induced increase in COX-2 expression that was mediated via PI3K/Akt pathway.

#### 4.4 | PKC is upstream PI3K and downstream p38

The results revealed that PI3K is downstream PKC, since PMA, an activator of PKC, did not inhibit the pump in presence of wortmannin. A possible mechanism of PKC activation of PI3K may be adopted from the work of Ziemba et al. (2016) who proposed that PKC phosphorylates the myristoylated alanine-rich C kinase substrate (MARCKS) protein, which sequesters PIP<sub>2</sub> molecules and by so doing reverses their sequestration and induces their release. The molecules act as docking sites and substrate for PI3K and thus recruit them to the membrane where they become functional.

The PMA-induced decrease in the ATPase activity was still observed when p38MAPK was inhibited, inferring that PKC is downstream p38 MAPK. Activation of PKC by p38 MAPK was reported in some works. Saurin et al. (2008) showed that activation of some PKC isoforms necessitates the sequential phosphorylation of three different serine residues in the hinge domain by respectively p38, GSK-3 $\beta$ , and by autophosphorylation.

#### 4.5 | FTY720P affects Na<sup>+</sup>/K<sup>+</sup> ATPase trafficking

FTY720P reduced the abundance of the ATPase molecules in the cell membrane as revealed by the imaging results, suggesting that FTY720P can affect trafficking of the pump between the cell membrane and intracellular pools. Such a translocation may contribute to the observed reduced activity.

Trafficking of the Na<sup>+</sup>/K<sup>+</sup> ATPase molecules occurs in order to adjust various cellular processes to changing cellular conditions and demands. Insulin for example was reported to induce an increase in the abundance of the  $\alpha$ 2 and  $\alpha$ 1 subunits of the pump in the cell membrane of rat heart muscle cells by recruiting them from an intracellular pool. This translocation was PI3K dependent and was reduced in diabetic rats resulting in lower number of  $\alpha$  subunits in the membrane (Rosta et al., 2009). Another kinase reported to be involved in the ATPase translocation is PKC, which reduced the number of pump molecules present in the cell membrane of epithelial A6-C1 cells by increasing fluid phase endocytosis (Beron et al., 1997). Intracellular Na<sup>+</sup> concentration is still another factor affecting pump trafficking. Blot-Chabaud et al. (1990) showed that a rise in intracellular sodium concentration in rat cortical collecting tubule (CCT) cells triggered an increase in the number of pump molecules present in the membrane. They provided experimental evidence that the observed upregulation of the Na<sup>+</sup>/K<sup>+</sup> ATPase is not due to an increase in its synthesis or a decrease in its degradation, but rather to a redistribution of inactive Na<sup>+</sup>/K<sup>+</sup> ATPase molecules from a latent intracellular pool to the plasma membrane of CCT cells.

In the previous work, we demonstrated that FTY720P down regulates the pump via PGE2 and nitric oxide. The latter is known to

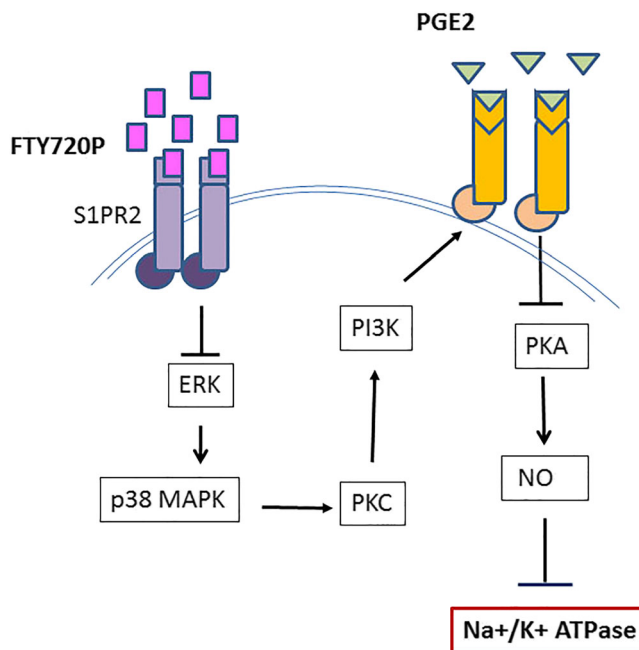


FIGURE 8 Proposed signaling pathway

regulate also protein trafficking by inhibiting exocytosis and enhancing endocytosis (Lowenstein, 2007).

Pump translocation may thus be dependent on PI3K, PKC, and NO, which are mediators identified in this work along the S1PR2 signaling pathway.

## 5 | CONCLUSIONS

It can be concluded that FTY720P downregulates the Na<sup>+</sup>/K<sup>+</sup> ATPase in Caco-2 cells by reducing its abundance in the cell membrane and its activity through a mechanism involving activation of S1PR2 probably through G $\alpha$ <sub>12/13</sub>, leading to ERK inhibition and activation of p38MAPK. The latter activates PKC, which in turn activates PI3K inducing PGE2 release. The prostaglandin, through EP3 activation and NO production, downregulates the Na<sup>+</sup>/K<sup>+</sup> ATPase. Figure 8 is a sketch of the proposed pathway.

Thus using FTY720P in the treatment of multiple sclerosis (Bravo et al., 2022) or inflammatory bowel disease is not without any side effect on colonic activities. A decrease in the ATPase activity is reflected by an alteration in electrolytes and water transport processes and would be probably be manifested as diarrhea.

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## CONFLICT OF INTEREST

The authors have no relevant financial or non-financial interests to disclose.

## AUTHOR CONTRIBUTIONS

All authors contributed to the study conception and design. Data collection and analysis were performed by Reem Rida and Rawad Hodeify. The manuscript was written by Sawsan Kreydiyyeh, reviewed and approved by the other authors.

## CONSENT FOR PUBLICATION

All authors read and approved the manuscript and its submission. No consent is needed from the University where the work was conducted.

## DATA AVAILABILITY STATEMENT

The manuscript has no associated data. All data are present in the manuscript.

## ORCID

Sawsan Kreydiyyeh  <https://orcid.org/0000-0002-4149-4081>

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