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Original Paper

Signaling Cascade Mediating the Effect of FTY720P on the Na^+/K^+ ATPase in LLC-PK1

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Key Words
FTY720P • Na^+/K^+ ATPase • LLC-PK1 • Calcium • Rho kinase • PI3K • NO

Abstract
Background/Aims: In renal ischemia, the Na^+/K^+ ATPase of the kidney epithelial cells translocates to intracellular compartments, resulting in altered kidney functions. Sphingosine-1-phosphate (S1P) was shown to play a protective role against this ischemic injury. Whether the sphingolipid targets the Na^+/K^+ ATPase is a possibility that has not been explored before. This work aims at investigating the effect of S1P on renal Na^+/K^+ ATPase using its analogue FTY720P and LLC-PK1 cells. ***Methods:*** The activity of the Na^+/K^+ ATPase was assayed by measuring the amount of inorganic phosphate liberated in presence and absence of ouabain, a specific inhibitor of the enzyme while its protein expression was studied by western blot analysis. ***Results:*** FTY720P increased the activity of the ATPase in a dose and time dependent manner, with a highest effect observed at 15 minutes and a dose of 80 nM. The protein expression was also increased. The stimulation of the Na^+/K^+ ATPase disappeared completely in presence of JTE-013, a specific blocker of S1PR2, as well as in presence of Y-27632, a Rho kinase inhibitor, BAPTA-AM, a Ca^{2+} chelator, wortmannin, a PI3K inhibitor, carboxy-PTIO, a scavenger for nitric oxide (NO), and KT 5823, a PKG inhibitor. CYM 5520, a S1PR2 agonist mimicked the effect of FTY720P. FTY720P increased the expression of p-Akt, a direct effector of PI3K, however, this increase disappeared when Rho kinase was inhibited, revealing that Rho kinase acts upstream PI3K. Glyco-SNAP-1, a NO donor, activated the pump in both presence and absence of wortmannin, indicating that PI3K is upstream NO. Interestingly, glyco-SNAP-1 and 8-bromo-cGMP, a PKG activator, exerted no effect on the Na^+/K^+ ATPase in absence of free Ca^{2+} revealing that the NO mediated effect is calcium-dependent. The involvement of calcium was further confirmed by the translocation of NFAT to the nucleus. The presence of verapamil or extracellular EGTA abolished the stimulatory effect of FTY720P, indicating that the source of calcium is extracellular. ***Conclusion:*** The results suggest that FTY720P activates sequentially S1PR2, Rho kinase, PI3K, leading to NO release and PKG stimulation. The latter phosphorylates calcium channels in the cell membrane, leading to calcium influx, and translocation of the ATPase units to the membrane.

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Introduction

The kidneys play a pivotal role in the regulation of blood composition, blood volume and blood pressure. Through selective reabsorption of metabolites and electrolytes, they maintain homeostasis and the constancy of the internal milieu. Many of the transport processes across the membrane of tubular cells are geared by the sodium gradient established by the Na^+/K^+ ATPase, known also as the Na^+/K^+ pump, which resides normally in the basolateral membranes. Renal ischemia impairs kidney functions, leading to acute kidney injury (AKI), and resulting in morbidity and mortality [1, 2]. When renal epithelial cells are energy-deprived, they lose their polarity [3-5], and the Na^+/K^+ ATPase translocates to intracellular compartments [6]. Regain of cell polarity and relocalization of the Na^+/K^+ ATPase in the plasma membrane are signs of recovery from renal ischemia [7].

Sphingosine-1-phosphate (S1P), a pleiotrophic lipid mediator, was found to play a protective role against ischemic renal injury IRI [8-11] and FTY720P an analogue of S1P, reduced renal injury in diabetic nephropathy [12].

Because S1P and the Na^+/K^+ ATPase are both implicated in renal ischemic injury, a cause effect relationship was suspected to exist between the sphingolipid and the ATPase. The current study was undertaken to test this hypothesis using the porcine kidney proximal tubule cell line LLC-PK1 as a model, and FTY720P as an analogue of S1P. The results revealed an FTY720P-induced increase in the activity of the ATPase via S1PR2, which leads to sequential activation of Rho kinase and PI3K, followed by NO release and PKG activation. Finally, we demonstrate that these changes are dependent on intracellular calcium.

Materials and Methods

Materials

FTY720P, anti-p-Akt1/2/3 (Ser473)-R rabbit polyclonal antibody, Akt1/2/3 (H-136) rabbit polyclonal antibody, goat anti-mouse horseradish peroxidase (HRP) conjugated IgG, anti-GAPDH mouse monoclonal antibody, KT5823, carboxy-PTIO, glycol-SNAP-1,8-bromo-cGMP were purchased from Santa Cruz Biotechnology, CA, USA. Goat anti-rabbit horseradish peroxidase (HRP) conjugated IgG and the protein ladder were purchased from Abcam, Cambridge, UK. (R)-3-Amino-(3-heylphenylamino)-4-oxobutylphosphonic acid (TFA salt, W146) was purchased from Avanti Polar Lipids, Inc.,Alabaster, Alabama. SEW 2871, CYM 5520, CYM 5541, JTE-013 and sterile dimethyl sulfoxide were obtained from TOCRIS Bioscience, Bristol, UK. CA10444 was obtained from Cayman Chemical Company, Michigan, USA. Y-27632 was procured from Cell Signaling Technology, Danvers, USA. Phorbol-12-myristate-13-acetate (PMA), Rp-Adenosine 3',5'-cyclic monophosphorothioate triethylammonium salt (RpcAMP), Calphostin C, Wortmannin, and BAPTA/AM were procured from Calbiochem, San Diego, USA. Biorad assay and protein reagent, nitrocellulose membranes, clarity western ECL substrate, were purchased from Bio-rad, California, USA. Porcine kidney cells, LLC-PK1 were purchased from American Type Culture Collection (ATCC). Anti-Na^+/K^+ ATPase α-1 Antibody, 2-Aminoethyldiphenyl borate (2-APB), and all other chemicals were obtained from Sigma, Chemical Co, St Louis Missouri, USA.

Culture and treatment of LLC-PK1 cells

LLC-PK1 cells were grown in DMEM supplemented with 10% FBS and 1% penicillin, in a humidified incubator (95% O_2, 5% CO_2) at 37 °C. Cells were treated with FTY720P at 85%-90% confluence after an overnight starvation. An equal amount of the vehicle was always added to the control group in each treatment.

Dose and Time response study on the effect of FTY720P on the activity of the Na^+/K^+ ATPase

LLC-PK1 cells were treated for15 minutes with different concentrations of FTY720P ranging from 0 to 750 nM. An equal amount of the vehicle DMSO was added to the control group. The cells were then washed with PBS buffer, lysed, homogenized, and spun for 30 minutes at 35000 g and 4 °C. The supernatant was collected and used to assay for the Na^+/K^+ ATPase activity or for western blot analysis. Proteins in the supernatant were quantified according to the Bradford method.

For the time-dependent study, LLC-PK1 cells were treated with 80 nM FTY720P for different time periods (0-4 hrs). The cells were then collected, treated as described above, and assayed for the Na^+/K^+ ATPase activity.

Type of S1PR involved in the signaling pathway of FTY720P

To determine the type of S1PR mediating FTY720P's effect on the Na^+/K^+ ATPase, S1PR1, S1PR2 and S1PR3 were individually blocked with their respective antagonists: W146 (10 μM), JTE-013 (1 μM) and CAY-10444 (17.4 μM). The blockers were added 30 minutes before FTY720P (80 nM, 15 min).

To confirm the type of S1PR involved, cells were also treated for 15 minutes with the respective agonists of S1PR1, S1PR2 and S1PR3: SEW2871 (100 nM [13]), CYM5520 (2.5 μM [14]), and CYM5541 (2 μM [14]).

Determination of the G protein coupled to the S1PR

S1P receptors are coupled to G_i, G_q or $G_{12,13}$ [15]. G_i is known to inhibit PKA. To investigate any role of G_i, cells were treated with a PKA inhibitor, RpcAMP (30 μM, [16]), 30 minutes prior to the addition of FTY720P or with a cell permeable cAMP analogue, dibutyryl-cAMP (dbcAMP: 10 μM [16]) for 15 minutes.

On the other hand, G_q activates PKC. The involvement of PKC was tested by treating the cells for 30 minutes, prior to FTY720P, with a PKC inhibitor, namely calphostin C (50 nM, [14]). In addition, the effect of phorbol 12-myristate 13-acetate (PMA: 100 nM, [14], 15 min), a PKC activator was investigated.

Finally, $G_{12/13}$ has Rho kinase as a downstream effector. To test for the involvement of $G_{12,13}$, cells were treated with a Rho kinase inhibitor, Y-27632 (10 μM, [17]) for 3 hours, prior to the addition of FTY720P.

Involvement of Rho kinase/PI3K pathway

Rho kinase was reported to activate PI3K [18], which in turn modulates the activity of the Na^+/K^+ ATPase [19]. The role of PI3K as a mediator was investigated by treating the cells, prior to FTY720P, with a PI3K inhibitor, wortmannin (100 nM, [20], 30min).

Cross talks between Rho kinases and PI3K have been recognized [21]. To position Rho kinase relative to PI3K, the protein expression of p-Akt, a downstream effector of PI3K, was determined by western blot analysis in cells in which Rho kinase was inhibited with Y-27632 (10 μM, 3hrs), before treatment with FTY720P.

Involvement of nitric oxide and its position relative to PI3K

The involvement of NO was investigated by treating the cells with carboxy-PTIO, a nitric oxide scavenger (30μM, [22]) for 30 minutes before treatment with FTY720P.

To locate NO relative to PI3K, the cells were treated for 15 minutes with glyco-SNAP-1 (4 μM, [22]), a nitric oxide donor, in the presence or absence of wortmannin (100 nM), a PI3K inhibitor added 30 minutes before glyco-SNAP-1.

Involvement of PKG

Guanylate cyclase is activated by NO and induces the production of cGMP, which in turn activates PKG. The involvement of the latter was investigated by determining the effect of FTY720P in cells, pretreated with KT-5823 (2.34 μM, [22]), a PKG inhibitor, 30 minutes prior to FTY720P.

Involvement of calcium

Nitric oxide and cGMP are suspected to play a role in calcium regulation. This hypothesis was tested by incubating the cells with a Ca^{2+} chelator, BAPTA-AM (20 nM, [15]) for 30 minutes prior to treatment with FTY720P.

NFAT1-GFP translocation was studied using a GFP-tagged NFAT1-GFP (Addgene #11107) [23]. LLCPK1 cells were plated on Poly-D-Lysine coated glass-bottomed dishes (MatTek) one day before co-transfection with1 μg NFAT1-GFP plasmid DNA using ViaFect™ Transfection Reagent. After 24 hours of transfection, cells were treated for 15 minutes with 80 nM FTY720P before fixation with 4% paraformaldehyde and then imaging with confocal microscope Zeiss LSM710. Nuclear GFP signal was quantified in individual cells using ImageJ (National Institutes of Health).

NFAT is a transcription factor that translocates from the cytoplasm to the nucleus when activated by dephosphorylation by the Ca2+-dependent phosphatase calcineurin. The involvement of calcium was investigated further by studying NFAT1-GFP nuclear translocation after FTY720P treatment.

To confirm that changes in intracellular calcium are induced by NO and PKG, cells were treated for 15 min with glyco-SNAP-1 (4 μM), a nitric oxide donor, or with 8-bromo-cGMP (0.5 mM) a cell permeable cGMP analogue, in presence of a Ca^{2+} chelator, BAPTA-AM (20 nM). The source of calcium was determined by blocking its release from intracellular stores with 2-aminoethoxydiphenyl Borate (2-APB) (60 μM, [15]), a blocker of IP3 channels [24] or by blocking its entry from the extracellular medium with verapamil (10 μM, [25]), an L-type calcium channel blocker, or with EGTA (0.5 mM) a calcium chelator.

Since APB may induce sometimes-extracellular calcium entry, its effect on the ATPase activity was investigated in the simultaneous presence of verapamil. Verapamil (10 μM) was added 30 minutes before APB, which was then applied for 15 minutes.

Finally, the involvement of calcium from intracellular stores in the FTY720P effect on the Na^+/K^+ ATPase was studied by inhibiting the sarcoplasmic Ca^{2+} ATPase (SERCA) with thapsigargin, blocking thus the pumping of calcium into the lumen of the endoplasmic reticulum (ER), inducing an increase in intracellular calcium levels, and eventually depletion of ER stores. Thapsigargin (1 μM, [26]) was applied 10 min before FTY720P.

The Na^+/K^+ ATPase activity assay

Treated cells were collected, washed, homogenized and spun as described before. The supernatant obtained was used to assay for the Na^+/K^+ ATPase activity. The protein concentration of each sample was adjusted to 0.5 μg/μL by addition of histidine buffer (150 mM, pH 7.4). Samples were incubated for 15 min at room temperature with 1% saponin added at a ratio of 1:4, followed by another incubation for 15 minutes with phosphatase inhibitors (2.7 mM pyrophosphate, 2.7 mM glycerophosphate). Aliquots were then taken from each sample and incubated for 15min at 37 °C in histidine buffer containing NaCl (121.5mM), KCl (19.6 mM), MgCl2 (3.92 mM), adenosine tri-phosphate (2.94 mM), in presence or absence of ouabain (1.47 mM), a specific inhibitor of the ATPase. 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 H, Shorr [27].

Membrane fractionation

Treated cells were washed with PBS scraped, passed through a 26-gauge needle 10 times, then through a 27-gauge needle for an additional ten times, and left on ice for 20min. The lysed cells were then spun at 720g for 5 min. The obtained supernatant was subjected to an additional 5 min centrifugation at 720g. The new supernatant obtained was spun for 10min at 10,000g. The supernatant was collected and centrifuged at 25,000g for 20min. The resultant pellet containing a crude membrane homogenate was used to assay for the protein expression of the Na^+/K^+ ATPase by western blot analysis. All centrifugation steps were conducted at 4 °C.

Western Blot analysis

Forty micrograms proteins from each sample homogenate were loaded and resolved on a 10% SDS polyacrylamide gel, transferred to a nitrocellulose membrane, and incubated overnight at 4 °C with a specific primary antibody for the Na^+/K^+ ATPase, p-Akt1/2/3 (Ser473)-R, Akt1/2/3 (H-136), or GAPDH. The membranes were then incubated with Goat anti-rabbit HRP conjugated secondary antibodies for 1 hour at room temperature. The signal was detected by chemiluminescence using Clarity ECL Substrate and its intensity was determined using a ChemiDocTM imaging system. GAPDH expression was used to check for equal loading. The bands were quantified and normalized to GAPDH using Image lab software. To assess for changes in the protein expression of the Na^+/K^+ ATPase in the membrane, the same steps were followed except that thirty microgram protein samples were taken from the crude membrane homogenate prepared as described before.

Statistical analysis

The data are reported as mean ± 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.

Results

Dose and time response study

LLC-PK1 cells treated for 15 minutes with different concentrations of FTY720P showed an increase in the activity of the Na^+/K^+ ATPase with a highest effect observed at 80 nM (Fig. 1A). FTY720P (80nM) applied for different time periods, exerted a maximal activation of the Na^+/K^+ ATPase at 15 minutes (Fig. 1B).

Protein expression of the Na^+/K^+ ATPase

FTY720P (80 nM) applied for 15 min increased the protein expression of the Na^+/K^+ ATPase in the membrane. This increase was almost of the same order of magnitude as the increase in the activity of the ATPase (Fig. 2).

Type of S1PR involved in the signaling pathway of FTY720P

Blocking S1PR1 with W146 (Fig. 3A), and S1PR3 with CAY-10444 (Fig. 3B), did not eliminate FTY720P's effect on the Na^+/K^+ ATPase. In addition, neither SEW2871, a S1PR1 agonist (Fig. 3C) nor CYM5541, a S1PR3 agonist (Fig. 3D) exerted any effect on the activity of the ATPase.

In presence of JTE-013, a S1PR2 antagonist, FTY720P's activation of the pump was not observed anymore (Fig. 4A), while CYM5520, a S1PR2 agonist induced a significant increase in the pump's activity (Fig. 4B).

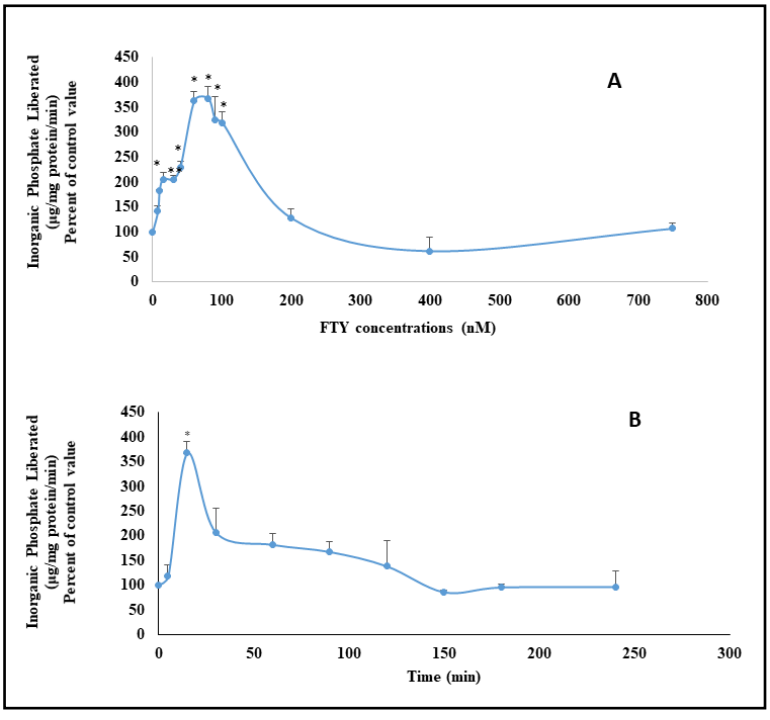


Fig. 1. Dose and time response study on the effect of FTY720P on the activity of the Na^+/K^+ ATPase in LLCPK1. A. The effect of FTY720P, applied for 15 minutes, on the activity of the Na^+/K^+ ATPase was dose dependent. B. FTY720P (80 nM) applied for 15 minutes modulates in a time dependent manner the activity of Na^+/K^+ ATPase. Values are means ±SEM of 3 observations. *significantly different from the control at P<0.01, **significantly different from the control at P<0.05 as indicated by ANOVA followed by Tukey Kramer test.

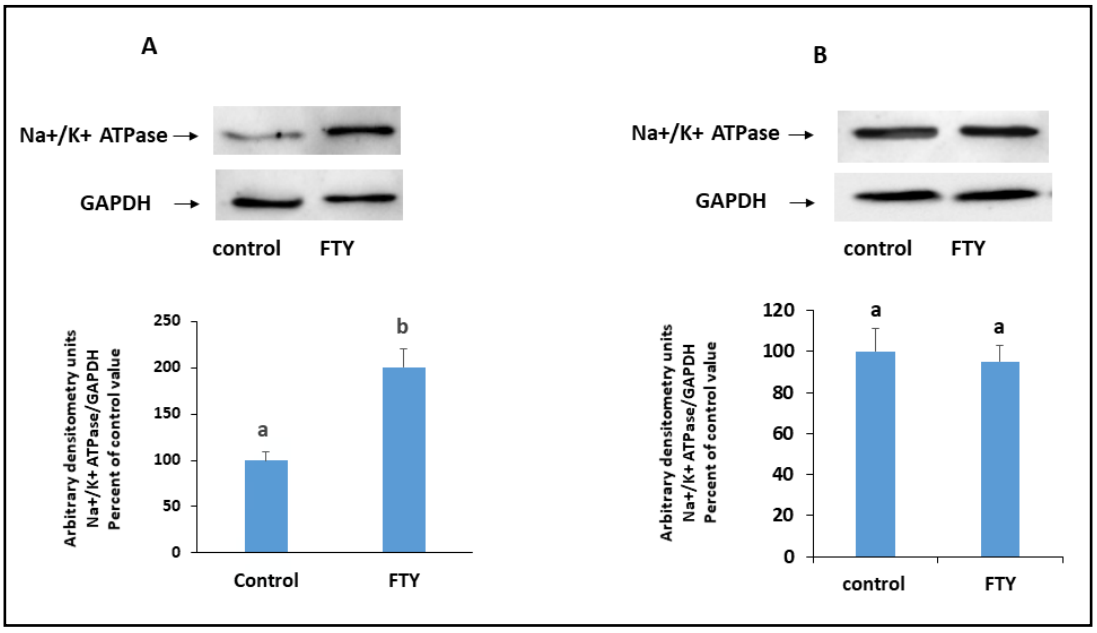


Fig. 2. Effect of FTY720P on the protein expression of the Na^+/K^+ ATPase. A. in total cell lysates and B. in membranes prepared from cells treated with FTY720P (80 nM) for 15 min. The bands are normalized to GAPDH. The blots are representative of an experiment repeated three times. The bars represent the densitometric analysis of the gels. Values are means ± SEM of at least 3 observations. Bars not sharing a common letter are considered significantly different from each other $p< 0.01$.

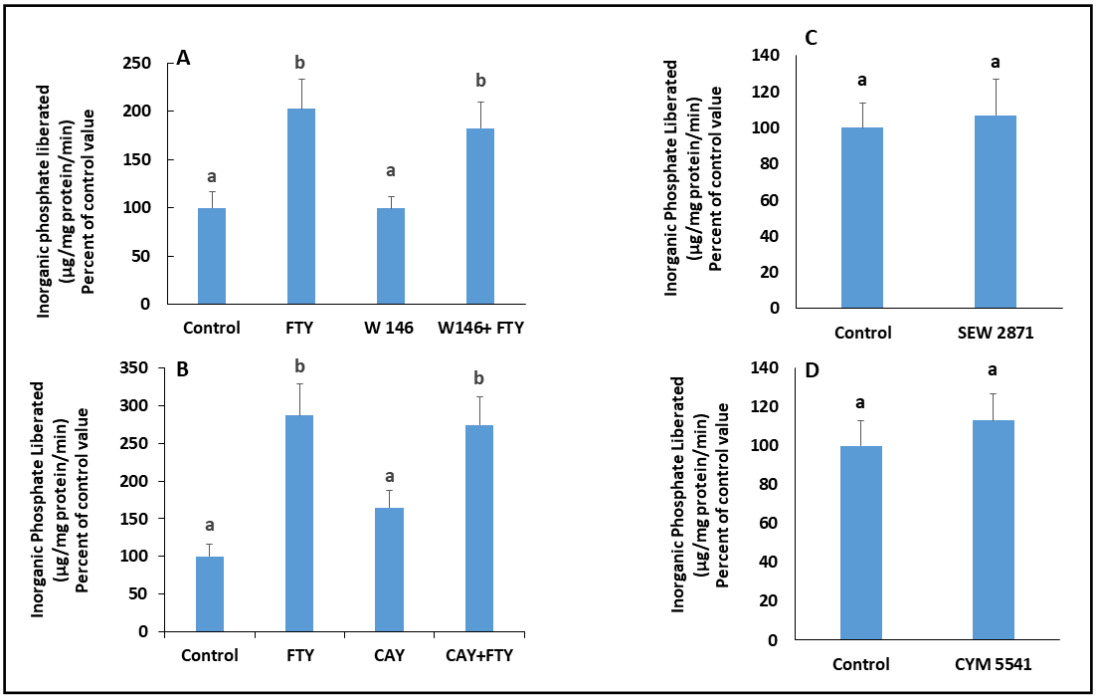


Fig. 3. Effect of antagonists and agonists of S1PR1 and S1PR3 on the activity of the Na^+/K^+ ATPase. Effect of FTY720P (80 nM, 15 min) on the Na^+/K^+ ATPase activity in presence of A. W146 (10 µM), B. Cay-10444 (17.4 µM), respective antagonists of S1PR1 and S1PR2, or in presence of C. SEW2871 (100 nM, 15 min). D.CYM5541 (2µM, 15 min), respective agonists forS1PR1 and S1PR3. Values are means ± SEM of 3 observations. Bars not sharing the same letter are considered significantly different from each other at $P<0.01$, as indicated by ANOVA followed by Tukey Kramer test. (FTY=FTY720P; CAY=CAY-10444).

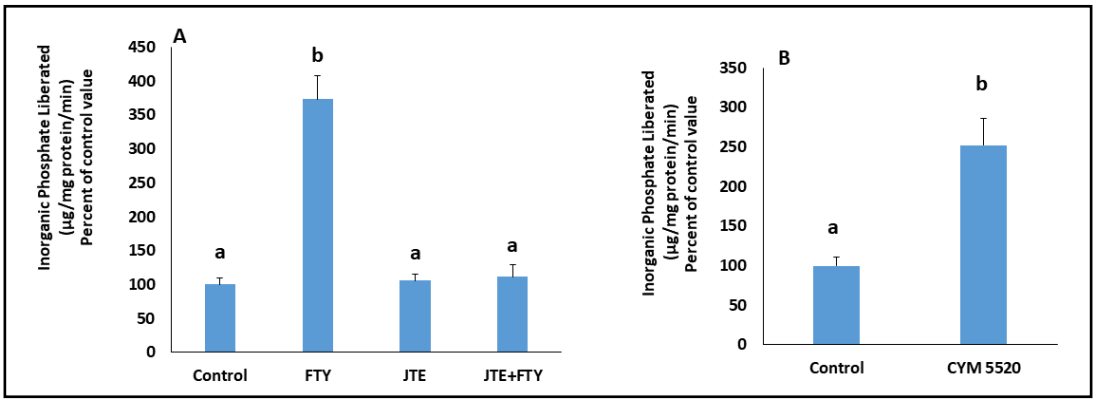


Fig. 4. Effect of an antagonist and agonist of S1PR2 on the activity of the Na^+/K^+ ATPase. Effect of FTY720P (80 nM, 15 min) in cells pre- treated with A. JTE-013 (1 µM, 15min), a S1PR2 antagonist, or B. CYM5520 2.5 µM, 15 min) a S1PR2 agonist. Values are means ± SEM of 3 observations. Bars not sharing a common letter are considered significantly different from each other at P<0.001, as indicated by ANOVA followed by Tukey Kramer test. (FTY=FTY720P, JTE=JTE-013).

Mediators of FTY720P's effect on the Na^+/K^+ ATPase

S1PR2 is coupled to $G_{i/o}$, G_q and $G_{12/13}$.

$G_{i/o}$ inhibits adenylyl cyclase, decreases the level of cAMP and consequently inhibits PKA. On the other hand, G_q activates PKC while $G_{12/13}$ acts via Rho kinase. Inhibition or activation of these kinases should respectively mimic or cancel the effect of FTY720P. The involvement of PKA was studied using a PKA inhibitor, RpcAMP, and a cell permeable analogue of cAMP, dibutyryl-cAMP (dbcAMP). RpcAMP did not mimic the effect of FTY720P on the pump (Fig. 5A) nor did dbcAMP abolish its stimulatory effect on the pump (Fig. 5B).

If the S1PR2s activated by FTY720P act via G_q, then they are expected to induce PKC activation. The activation of the Na^+/K^+ ATPase was still observed in presence of calphostin C (Fig. 6A), a PKC inhibitor, and phorbol 12-myristate 13-acetate (PMA), a PKC activator, did not exert any effect on the ATPase (Fig. 6B).

$G_{12/13}$ is another G protein to which S1PR2 could be coupled, and when activated induces activation of Rho kinase. Treating the cells with a Rho kinase inhibitor, Y-27632, abolished the stimulation induced by FTY720P (Fig. 7).

Rho kinase activates PI3K

Inhibiting PI3K with wortmannin eliminated the activation of the Na^+/K^+ ATPase induced by FTY720P on (Fig. 8A).

The expression of p-Akt, a known target of PI3K, increased following treatment with FTY720P. However, it came back to the control value when Rho kinase was inhibited with Y-27632 (Fig. 8B). On the other hand, the expression of total Akt was not affected by FTY720P (Fig. 8C).

Nitric oxide is involved in FTY720P mediated effect and is downstream PI3K

The stimulatory effect of FTY720P was abolished by the NO scavenger carboxy-PTIO (Fig. 9A) and mimicked by Glyco-SNAP-1, a nitric oxide donor, even in presence of wortmannin, the PI3K inhibitor (Fig. 9B).

Nitric oxide is known to induce cGMP production and PKG activation. Inhibiting PKG with KT5823 (2.34 µM) eliminated the effect of FTY720P on the Na^+/K^+ ATPase (Fig. 9C).

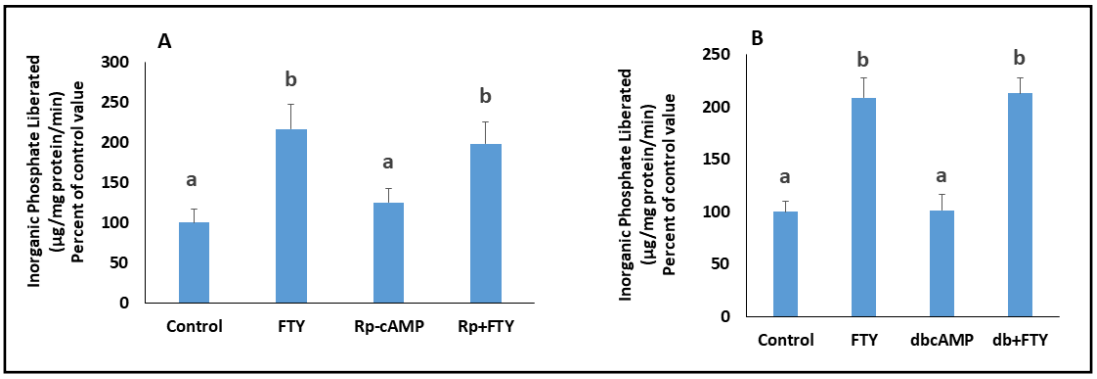


Fig. 5. Effect of FTY720P (80nM, 15 minutes) on the Na^+/K^+ ATPase activity in presence of A. RpcAMP (30 µM) or B. dbcAMP (10 µM), a respective PKA inhibitor and activator. Values are means ± SEM of 3 observations. Bars not sharing a common letter are considered significantly different from each other at P<0.001, as indicated by ANOVA followed by Tukey Kramer test. (FTY = FTY720P, Rp = RpcAMP).

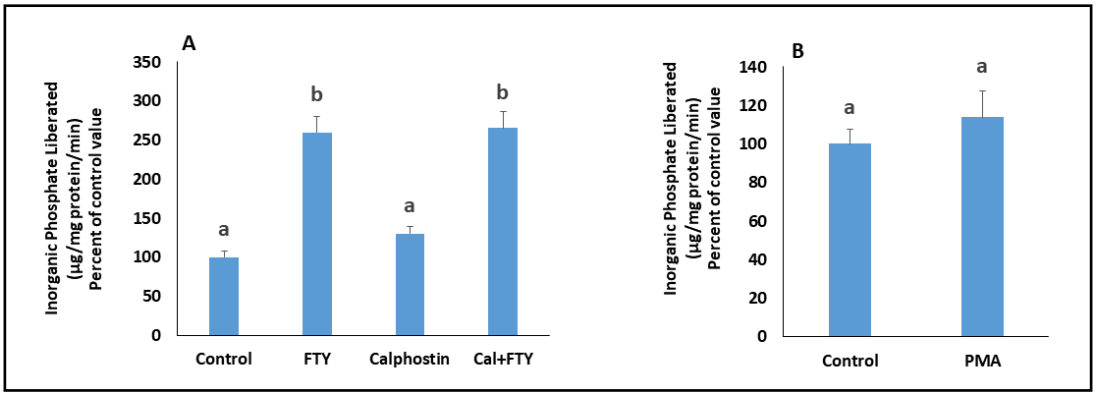
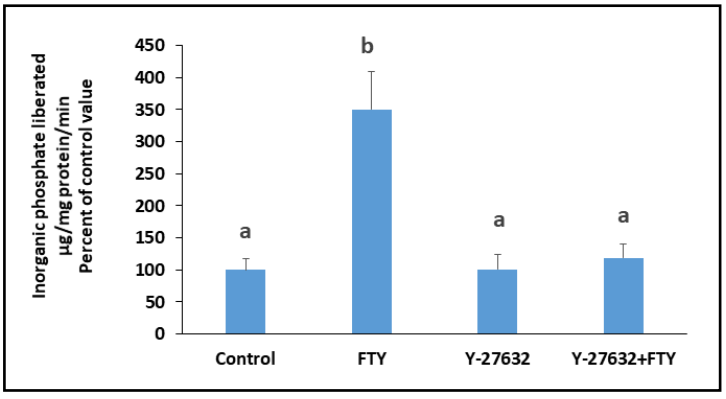


Fig. 6. A. Effect of FTY720P (80 nM, 15 min) on the Na^+/K^+ ATPase activity in presence of Calphostin C (50 nM), a PKC inhibitor. B. Effect of PMA (100 nM, 15 mins), a PKC activator on the Na^+/K^+ ATPase. Values are means ± SEM of 3 observations. Bars not sharing a common letter are considered significantly different from each other at P<0.001, as indicated by ANOVA followed by Tukey Kramer test. (FTY=FTY720P, Cal=Calphostin C).

Fig. 7. Effect of FTY720P (80 nM, 15 minutes) on the Na^+/K^+ ATPase activity in presence of Y-27632 (10 µM), a Rho kinase inhibitor. Values are means ± SEM of 3 observations. Bars not sharing a common letter are considered significantly different from each other at P<0.001, as indicated by ANOVA followed by Tukey Kramer test.



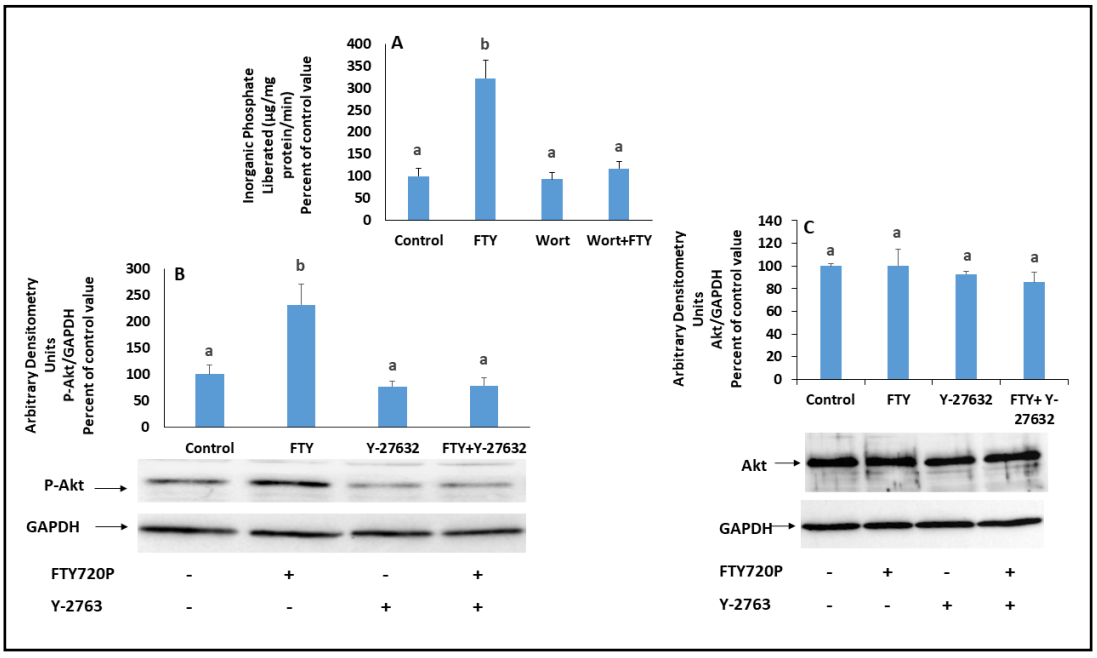


Fig. 8. A. Effect of FTY720P (80 nM, 15 minutes) on the Na^+/K^+ ATPase activity in presence of wortmannin (100 nM), a PI3K inhibitor. Values are means ± SEM of 3 observations. Bars not sharing the same letter are considered significantly different from each other at P<0.001, as indicated by ANOVA followed by Tukey Kramer test. (FTY=FTY720P, Wort=Wortmannin). Effect of FTY720P (80 nM, 15 minutes) in presence of Y-27632 (10 µM), a Rho kinase inhibitor on the protein expression of B. p-Akt, and C. total Akt. The bands are normalized to GAPDH. Bars represent densitometric analysis of the gels. Values are means ± SEM of at least 3 observations. Bars not sharing a common letter are considered significantly different from each other p< 0.01.

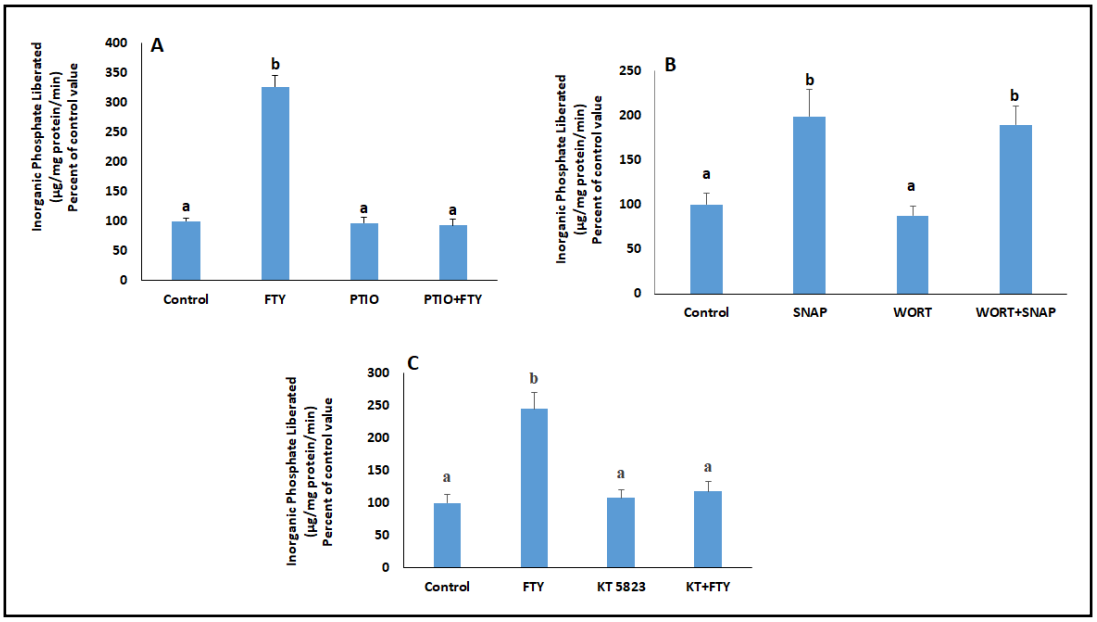


Fig. 9. A) Effect of FTY720P (80 nM, 15 min) on the Na^+/K^+ ATPase activity in presence of carboxy-PTIO (30 nM). B) Effect of Glyco-SNAP-1 (4 µM, 15 min) on the Na^+/K^+ ATPase in presence of wortmannin (100 nM). Values are means ± SEM of 3 observations. Bars not sharing a common letter are considered significantly different from each other at P<0.001, as indicated by ANOVA followed by Tukey Kramer test.

Calcium is involved in the signaling pathway and is downstream nitric oxide

An increase in cytosolic calcium can be the result of extracellular calcium influx through membrane channels or calcium release from the endoplasmic reticulum through IP3 receptors.

The translocation of the transcription factor NFAT from the cytosol to the nucleus is a calcium dependent process. Confocal microscopy revealed a significant increase in nuclear fluorescence in cells expressing GFP-tagged NFAT1 and treated with FTY720P (Fig. 10), suggesting involvement of calcium in the FTY720P signaling pathway.

In presence of BAPTA-AM, a Ca^{2+} chelator, the stimulatory effect of FTY720P on Na^+/K^+ ATPase disappeared (Fig. 11A). Similary, the effect of Glyco-SNAP-1 and and 8-Br-cGMP, a cell permeable analogue of cGMP, were not manifested in presence of BAPTA-AM. (Fig. 11B and C). FTY720P did not exert any effect on the ATPase when PKG, the target of cGMP, was inhibited with KT 5823 (Fig. 11D).

Source of calcium

Chelating extracellular calcium with EGTA (Fig. 12A) as well as blocking L-type calcium channels with verapamil (Fig. 12B), both abrogated the stimulatory effect of FTY720P, suggesting an extracellular source for the increase in cytosolic calcium.

Cells treated with FTY720P in presence of 2-aminoethoxydiphenyl Borate (2-APB) (60 µM), a blocker of IP3 receptors, exhibited an unexpectedly, higher activity of the Na^+/K^+ ATPase that disappeared in presence of verapamil (Fig. 12C), suggesting that 2-ABP at the used dose, triggered calcium entry through plasma membrane channels.

The inhibition of the sarco/endoplasmic reticulum Ca ATPase (SERCA) with thapsigargin did not have any effect on the activity of the Na^+/K^+ ATPase and in its presence, FTY720P exerted its full stimulatory effect when the cells were treated in a normal medium. The effect of FTY720P, however, did not appear when cells were treated in calcium-free PBS (Fig. 13).

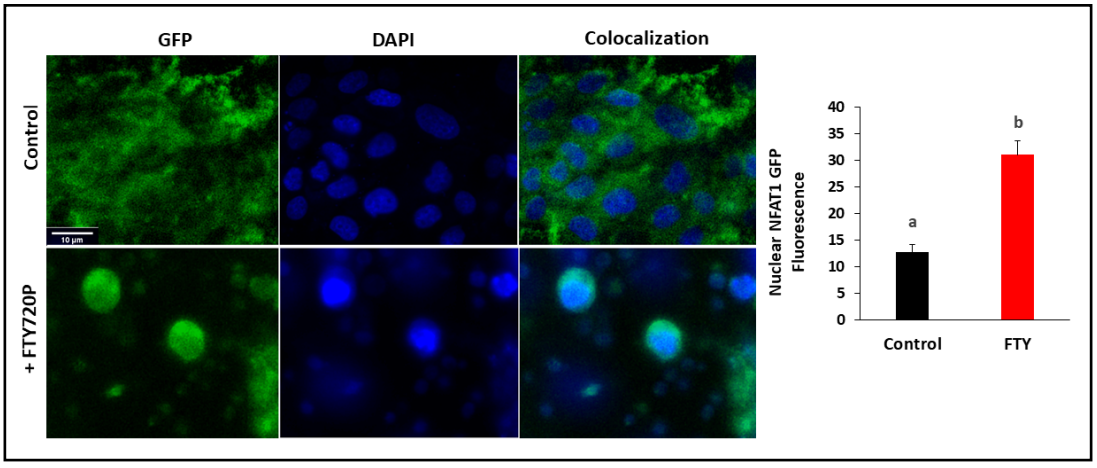


Fig. 10. Fluorescent imaging demonstrating translocation of NFAT1-GFP in LLCPK1 treated with FTY720P. Cells were transfected with 1 µg NFAT1-GFP plasmid using ViaFect™ Transfection Reagent and treated next day with 80nM FTY720P for 15 minutes. Cells were fixed with 4% paraformaldehyde and stained with DAPI. A. Representative fluorescent images of cells using confocal microscope Zeiss LSM710. B). B. Quantification of nuclear NFAT1-GFP using ImageJ. Values are means ± SEM. Bars not sharing a common letter are considered significantly different from each other at $p<0.01$.

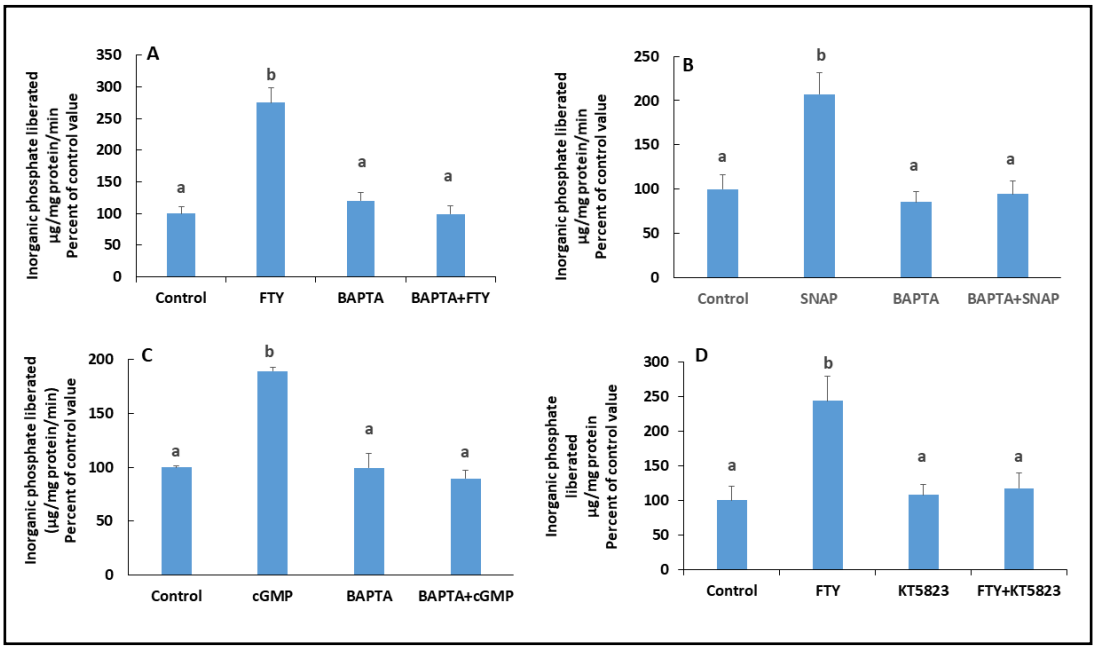


Fig. 11. Effect of A. FTY720P (80 nM, 15 min), B. Glyco-SNAP-1 (4 µM, 15 min) and C. 8-bromo-cGMP (0.5 mM, 15 min) on the Na^+/K^+ ATPase activity in presence of BAPTA-AM (20 nM), a Ca^{2+} chelator. D. Effect of FTY720P (80 nM, 15 min), in presence of KT 5823 (2.34 µM), a PKG inhibitor, on the Na^+/K^+ ATPase activity. Values are means ± SEM of 3 observations. Bars not sharing the same letter are considered significantly different from each other at P<0.001, as indicated by ANOVA followed by Tukey Kramer test. (FTY=FTY720P, BAP=BAPTA-AM).

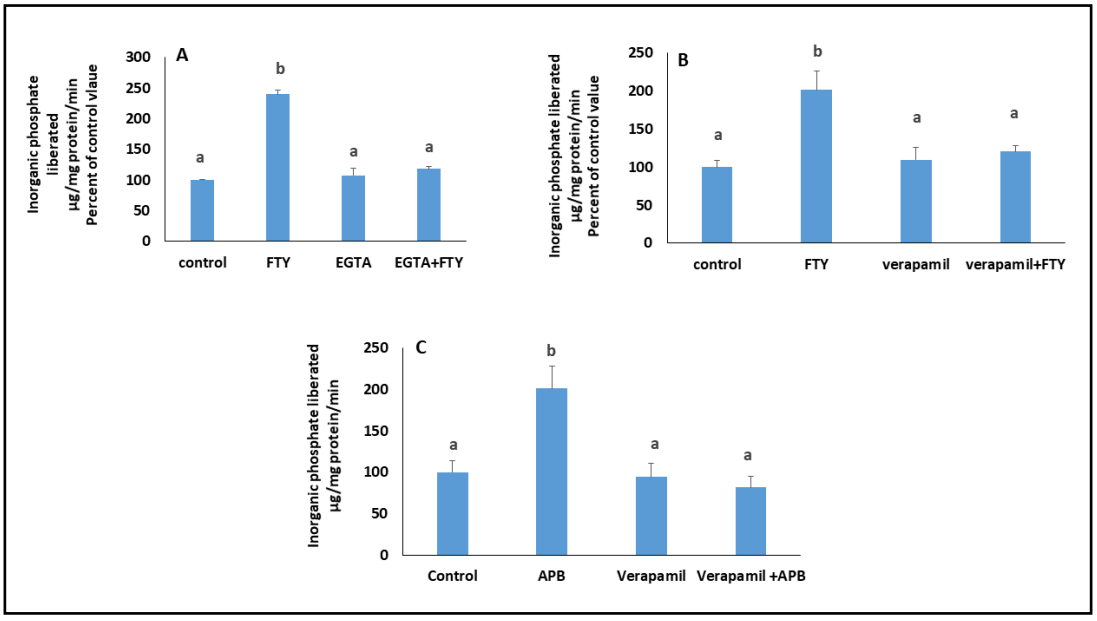


Fig. 12. Effect of A. Extracellular EGTA, B. verapamil and C. 2-APB in presence of verapamil on the activity of the Na^+/K^+ ATPase. Values are means ± SEM of 3 observations. Bars not sharing the same letter are considered significantly different from each other at P<0.001, as indicated by ANOVA followed by Tukey Kramer test.

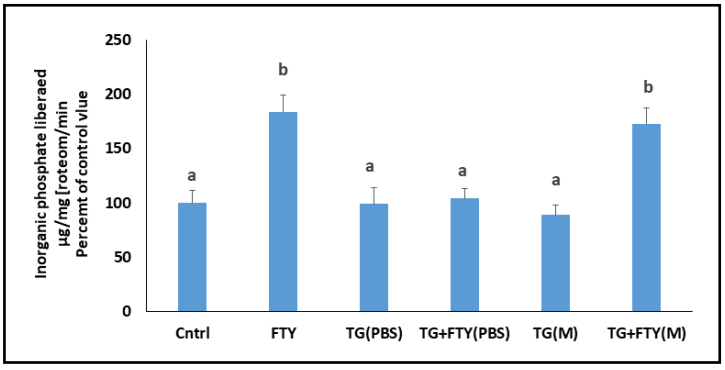


Fig. 13. Effect of FTY720P on the activity of the Na^+/K^+ ATPase in presence of thapsigargin, a SERCA inhibitor, in cells treated in a normal medium (M) or PBS. Values are means ± SEM of 3 observations. Bars not sharing the same letter are considered significantly different from each other at P<0.001, as indicated by ANOVA followed by Tukey Kramer test.

Discussion

The reabsorption of many solutes in the kidney depends on proper functioning of the Na^+/K^+ ATPase which establishes the sodium gradient needed to gear all secondary active transport processes [28]. Any alteration in the Na^+/K^+ ATPase's activity is expected to impair kidney function [29] and result in kidney failure. The ATPase is down regulated in renal ischemic reperfusion injury (IRI) [30], and FTY720, which is phosphorylated in the cell to the S1P analogue, FTY720P, was shown to confer protection against renal IRI, a protection mediated via activation of S1PR1 and a decrease in inflammatory cell infiltration [31]. This protective effect was however, still manifested in animals lacking B and T lymphocytes when treated with FTY720P or a selective S1P1R agonist raising the possibility of a direct effect on the proximal tubule cells [32].

In fact, most of the information related to the protective role of S1P or FTY720P comes from experiments conducted on whole animals. Thus, it remains possible that their effect on the kidney may be indirect and mediated through some humoral factors, hormones or cytokines released by non-kidney cells. This work was undertaken to investigate if FTY720P exerts any direct effect on proximal tubule cells and specifically on their Na^+/K^+ ATPase, which is downregulated during kidney injury, using the *in vitro* model of LLC-PK1 cells.

Dose and time response studies revealed a stimulatory action of FTY720P on the Na^+/K^+ ATPase, with the highest effect observed at a dose of 80 nM applied for 15 minutes. Higher concentrations of FTY720P restored the activity of the Na^+/K^+ ATPase back to control levels probably as a result of an activation, at higher doses, of different S1PRs with different binding affinities. FTY720P did not alter the activity of the ATPase when the cells were treated for longer time period, which may be due to desensitization or internalization and degradation of the receptors [33]. Such a dose and time dependent effect of FTY720P has been observed before in Caco-2 cells [34] and in HepG2 cells [35].

The observed increase in the activity of the Na^+/K^+ ATPase may be due to an increase in the number of the Na^+/K^+ ATPase molecules or to an increase in their specific activity. Western blot analysis revealed a higher membrane protein expression of the ATPase at 15 min, which could not be due to an increase in its synthesis within this short time period and is probably due to a translocation of the ATPase units from intracellular compartments to the membrane. This is supported further by the results of the western blot analysis conducted on whole cell lysates which revealed no change in the protein expression of the Na^+/K^+ ATPase.

FTY720P exerts its effect via five types of receptors (S1PR1-5) [36-38]. Blocking S1PR1 and S1PR3 with respectively W146 and CAY-10444 did not abolish the stimulatory effect of FTY720P suggesting that these two receptors are not involved. This was confirmed further when no change in the activity of the Na^+/K^+ ATPase were observed upon cell treatment with SEW 2871 and CYM 5541, respective agonists of S1PR1 and S1PR2.

Treating the cells however, with JTE-013, a blocker of S1PR2 abrogated completely the effect of FTY720P implying that the latter acts only via S1PR2. Had another additional

receptor been involved, then a partial stimulatory effect would have still remained in presence of JTE-013. This stimulatory effect was mimicked by CYM 5520, a S1PR2 agonist, confirming the involvement of S1PR2. Such a role of FTY720P in modulating the activity of the Na^+/K^+ ATPse has been observed before [34].

S1PR2 couples to $G_{i/o}$, G_q or $G_{12/13}$. Signaling via $G_{i/o}$ leads to adenylate cyclase inhibition and a reduction in the level of cAMP and PKA activity [15]. Inhibiting PKA with Rp-cAMp did not imitate the effect of FTY720P nor did dbcAMP counteract its stimulatory effect indicating that FTY720P does not act via G_i.

G_q signals through PKC [15], a kinase known to play a role in the trafficking of the Na^+/K^+ ATPase and modulation of its activity [39-44]. In presence of Calphostin C, a PKC inhibitor, FTY720P still exerted its usual stimulatory effect on the Na^+/K^+ ATPase while PMA, a PKC activator did not have any significant effect implying that PKC is not a mediator in FTY720P's signaling pathway, thus ruling out the involvement of G_q.

The third type of G protein that couples to S1PR2 is $G_{12/13}$, which activates Rho kinase. Y-27632, a Rho kinase inhibitor, abolished the effect of FTY720P, and the activity of the pump went back to control levels, suggesting that FTY720P acts via Rho kinase. Activation of the RhoA pathway was previously shown to induce trafficking between plasma membrane and intracellular compartments [45]. It induced exocytosis of the Na^+/K^+ ATPase in Alveolar Epithelial Cells [46] and was involved in the regulation and localization of the Na^+/K^+ ATPase in renal epithelial cells [47].

Another molecule involved in trafficking is PI3K [48]. The results confirmed its role as a downstream intermediate in FTY720P signaling, since no change in the activity of the ATPase was observed when it was inhibited with wortmannin. In fact, Serhan et al. [20] reported an inhibition of the pump and a decrease in its abundance following activation of PI3K by insulin in Caco-2 cells. The literature also reports an increase in the activity and expression of the Na^+/K^+ ATPase upon activation of the PI3K pathway in rats treated with estradiol [49].

PI3K acts by activating Akt [50] and enhancing its phosphorylation. The expression of p-Akt increased following treatment with FTY720P, but was not manifested when Rho kinase was inhibited with Y-27632 inferring that PI3K/Akt act downstream Rho kinase. Similar results were reported in endothelial cells where Rho kinase induced their motility via activation of PI3K/Akt [51].

Nitric oxide was shown to modulate the activity of the Na^+/K^+ ATPase [52]. Whether FTY720P exerts its effect on the ATPase via NO was a question that needed to be addressed. Carboxy-PTIO, a nitric oxide scavenger, blocked the effect of FTY720P on the ATPase while glyco-SNAP-1, a nitric oxide donor, induced as expected, a stimulation that was maintained in presence of wortmannin, a PI3K inhibitor, indicating that NO is involved and acts downstream PI3K. In fact, many works reported activation of nitric oxide synthase (NOS) by phosphorylation by the serine threonine protein kinase B or Akt [53], which is activated by PI3K, leading to NO production [54-56].

Nitric oxide signals through soluble guanylate cyclase/cGMP/PKG [57]. The data showed that this pathway is involved in the effect of FTY720P on the ATPase since in presence of KT 5823, a PKG inhibitor, the activity of the Na^+/K^+ ATPase was restored back to control values.

The literature reports a role of cGMP in intracellular calcium regulation [58, 59]. Since calcium is involved in intracellular trafficking [60]and since the ATPase activation is suspected to result from the translocation of its units from intracellular compartments to the membrane, the involvement of calcium in the response to FTY720P was investigated using a Ca^{2+} chelator, BAPTA-AM. In presence of BAPTA-AM, neither FTY720P nor the NO donor Glyco-SNAP-1, or 8-bromo-cGMP, exerted any effect on the Na^+/K^+ ATPase confirming the suspected role of calcium in modulating the ATPase activity, and revealing its position downstream NO and cGMP.

Further confirmation of a role for calcium in the signaling pathway came from the results with NFAT, which showed translocation of this transcription factor from the cytoplasm to the nucleus, a process that occurs as a result of its dephosphorylation by the Ca2+-dependent phosphatase calcineurin [61].

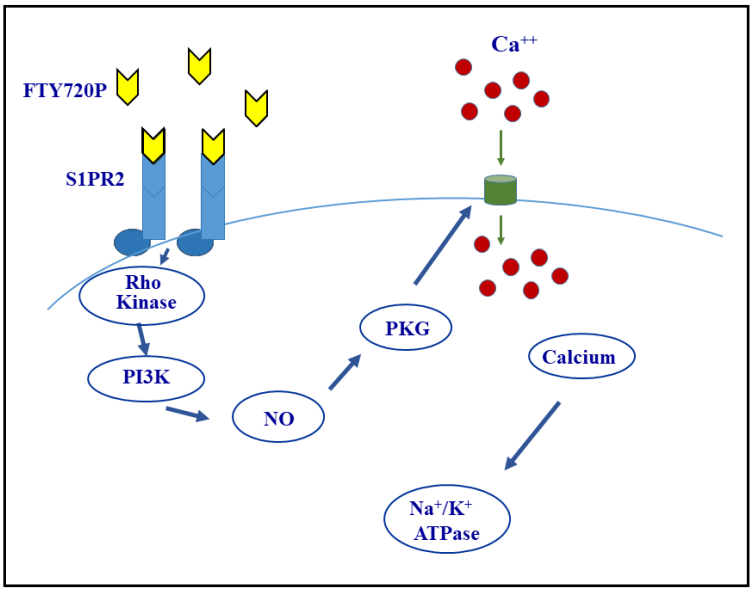


Fig. 14. Signaling pathway activated by FTY720P.

Cyclic GMP may regulate intracellular calcium level via PKG. The latter could phosphorylate and activate calcium channels in the cell membrane or the membrane of ER [62-64]. To determine if the calcium implicated in the signaling pathway is derived from intracellular stores, IP3 receptors were blocked with 2-APB. Opposite to our expectations, a significant increase in the activity of the ATPase was observed, that disappeared in the simultaneous presence of verapamil, a blocker of the membrane calcium channels. The results imply that hindering extracellular calcium entry abolished the effect of 2-APB. Similarly, chelating extracellular calcium with EGTA or preventing its entry with verapamil, abolished the stimulatory effect of FTY720P on the ATPase indicating that the source of cytosolic calcium is extracellular.

The dose-dependent differential activation of calcium entry by 2-APB was previously reported [65]. Importantly, although doses greater than 30 µM were shown to inhibit store operated calcium entry, such doses can induce calcium entry through Orai3 channels. Our results suggest that 2-APB induces calcium entry and consequently enhances ATPase activity.

Blocking SERCA with thapsigargin is expected to prevent calcium refilling of ER stores and to eventually lead to their calcium depletion. Thapsigargin did not interfere with the effect of FTY720P, however treating the cells in PBS abolished completely the FTY720P-induced stimulation of the ATPase in presence or absence of thapsigargin, reaffirming that the source of cytosolic calcium is extracellular.

Upon synthesis, the Na^+/K^+ ATPase should translocate to the basal membrane to perform its functions properly. Rho kinase was shown to play a major role in the trafficking process [66], which is regulated by calcium and involves reorganization of the cytoskeleton [67, 68].

The results obtained support a signaling pathway in which FTY720P activates S1PR2, which induces through the Rho pathway, activation of PI3K, NO release, production of cGMP and PKG activation. The latter may phosphorylate and gate open calcium channels in the cell membrane, leading to increase in cytosolic calcium, and translocation of the ATPase units to the membrane. Fig. 14 is a scheme summarizing the signaling pathway.

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Author Contributions

SK and RH contributed to the study conception and design. CK contributed to data collection and analysis. The manuscript was written by SK, reviewed and approved by the other authors.

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Statement of Ethics
The authors have no ethical conflicts to disclose.

Disclosure Statement

The authors declare that no conflicts of interest exist.

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