

RESEARCH ARTICLE

# Epinephrine modulates Na<sup>+</sup>/K<sup>+</sup> ATPase activity in Caco-2 cells via Src, p38MAPK, ERK and PGE2

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## Abstract

Epinephrine, a key stress hormone, is known to affect ion transport in the colon. Stress has been associated with alterations in colonic functions leading to changes in water movements manifested as diarrhea or constipation. Colonic water movement is driven by the Na<sup>+</sup>-gradient created by the Na<sup>+</sup>/K<sup>+</sup>-ATPase. Whether epinephrine acts via an effect on the Na<sup>+</sup>/K<sup>+</sup>-ATPase hasn't been studied before. The aim of this work was to investigate the effect of epinephrine on the Na<sup>+</sup>/K<sup>+</sup>-ATPase and to elucidate the signaling pathway involved using Caco-2 cells as a model. The activity of the Na<sup>+</sup>/K<sup>+</sup>-ATPase was assayed by measuring the amount of inorganic phosphate released in presence and absence of ouabain, a specific inhibitor of the enzyme. Epinephrine, added for 20 minutes, decreased the activity of the Na<sup>+</sup>/K<sup>+</sup>-ATPase by around 50%. This effect was found to be mediated by α2 adrenergic receptors as it was fully abolished in the presence of yohimbine an α2-blocker, but persisted in presence of other adrenergic antagonists. Furthermore, treatment with Rp-cAMP, a PKA inhibitor, mimicked epinephrine's negative effect and didn't result in any additional inhibition when both were added simultaneously. Treatment with indomethacin, PP2, SB202190, and PD98059, respective inhibitors of COX enzymes, Src, p38MAPK, and ERK completely abrogated the effect of epinephrine. The effect of epinephrine did not appear also in presence of inhibitors of all four different types of PGE2 receptors. Western blot analysis revealed an epinephrine-induced increase in the phosphorylation of p38 MAPK and ERK that disappeared in presence of respectively PP2 and SB202190. In addition, an inhibitory effect, similar to that of epinephrine's, was observed upon incubation with PGE2. It was concluded that epinephrine inhibits the Na<sup>+</sup>/K<sup>+</sup>-ATPase by the sequential activation of α2 adrenergic receptors, Src, p38MAPK, and ERK leading to PGE2 release.

## OPEN ACCESS

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## Introduction

Stress, whether physical or mental, is a ubiquitous condition that is part of our everyday life. When confronted with potential stressors, the brain triggers a cascade of physiological reactions, known as the “fight or flight response”, to ensure the individual's survival and adaptation to the threatening events [1]. Neural inputs from the brain stimulate the hypothalamus to

release CRH (corticotrophin releasing hormone) which, in turn, activates both the sympathetic-adrenal medulla and pituitary-adrenal cortex axes, resulting in the respective release of the primary stress hormones: epinephrine and cortisol into the blood stream [2]. Together these hormones trigger the physiological deviations from homeostasis observed in the different systems of the body (cardiovascular, immune, endocrine, reproductive, respiratory, etc. . .) during the acute stress response [1]. A key target of the stress reaction appears to be the gastrointestinal tract (GI) whereby the prevalence and the severity of several GI disorders were found to correlate with anxiety, depression, and neuroticism [3]. Among the various GI diseases, the role of stress in the pathophysiology of irritable bowel syndrome (IBS) has been extensively studied [4]. IBS is considered one of the most prominent chronic gastrointestinal disorders, and is mainly characterized by abdominal pain and discomfort due to either frequent diarrhea or constipation [5]. Epinephrine, a key stress hormone, was reported to affect water movement across the epithelium of certain tissues such as the human eye [6,7], lungs [8], and kidneys [9]. Nonetheless, a potential role of epinephrine, in the alteration of colonic water movement and the development of IBS symptoms, or even their exacerbation, has not been investigated before.

Water movement across epithelial layers of the colon is governed by the Na<sup>+</sup> gradient created by the Na<sup>+</sup>/K<sup>+</sup>-ATPase. By pumping 3Na<sup>+</sup> ions to the outside of the cell in exchange for 2K<sup>+</sup> ions to the inside, the Na<sup>+</sup>/K<sup>+</sup>-ATPase establishes and maintains a low intracellular Na<sup>+</sup> concentration which drives Na<sup>+</sup> ions to flow down their electrochemical gradient from the lumen into the cytosol. This Na<sup>+</sup> diffusion generates osmotic forces that cause water molecules to follow across the plasma membrane. Consequently, an alteration in the activity of the Na<sup>+</sup>/K<sup>+</sup>-ATPase was found to modify the direction and rate of net water transport, as detected in the intestines of deoxycorticosterone acetate- injected mice [10], in the ileum of methylprednisolone -pretreated rats [11], in rat proximal tubular cells following high Na<sup>+</sup>-diet [12], and in rat brain during acute cerebral ischemia [13].

In an attempt to understand the relation between the stress reaction and colonic water movement, we aimed to study the effect of epinephrine on the activity of the Na<sup>+</sup>/K<sup>+</sup>-ATPase in colon adenocarcinoma cells (CaCo-2), and to elucidate its underlying mechanism of action. Identifying the different mediators involved in the effect of epinephrine on the ATPase would help in finding therapeutic strategies that target them and relieve the undesirable effects of stress on colonic functions.

## Materials and methods

### Materials

Dulbecco's Minimal Essential Medium (DMEM) with 4500mg glucose/L and pyridoxine HCl, Fetal Bovine Serum(FBS), Trypsin-EDTA, Penicillin/Streptomycin(PS), 10x Phosphate Buffered Saline (PBS) without calcium and magnesium, (-)-Epinephrine, L-Ascorbic Acid, N6,2'-O-Dibutyryl adenosine 3',5'-cyclic monophosphate sodium salt (dbcAMP), Adenosine 5'-triphosphate disodium salt(ATP), ouabain, prostaglandin E2 (PGE2), SC 19220, indomethacin, DL-propranolol, yohimbine, prazosin, and PF-04418948 were purchased from Sigma, Chemical Co, St. Louis Missouri, USA. PP2, PD98059 and SB202190, respective inhibitors of Src, MEK/ERK and p38MAPK, were obtained from Merck Millipore, MA, USA.

The human colon carcinoma cell line (CaCo-2) was purchased from American Type Culture Collection (ATCC), VA, USA.

Rabbit anti-ERK1/2 polyclonal antibody and rabbit anti-p-p44/42 MAPK (ERK1/2) monoclonal antibody were purchased respectively from Promega, WI, USA, and Cell Signaling, MA, USA. Rabbit anti-p38 $\alpha$ , anti-p-p38 $\alpha$  polyclonal antibodies, anti-rabbit IgG horse raddish

peroxidase (HRP) conjugated, L-798106 and GW 627368X were purchased from Santa Cruz, CA, USA. Rabbit anti-Src and anti-pSrc polyclonal antibodies as well as anti GAPDH monoclonal antibody were purchased from Cell signaling, MA, USA. Protease inhibitors cocktail tablets were purchased from Boehringer Mannheim, Germany. Clarity ECL Substrate, Nitrocellulose membranes and Bio-Rad protein assay reagent were purchased from Bio-Rad, California, USA.

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

## Methods

**Cell culture of CaCo-2 cells.** CaCo-2 cells were used at passages 25–32. They were grown, at a density of 1200,000/well, on 100mm culture dishes in DMEM containing 4500 mg L<sup>-1</sup> Glucose, sodium pyruvate, 1% Penicillin (100 µg mL<sup>-1</sup>), streptomycin (100 µg mL<sup>-1</sup>), 10% FBS, in a humidified incubator (95% O<sub>2</sub>, 5% CO<sub>2</sub>) at 37°C. Cells were always treated at 80–90% confluence.

**Effect of epinephrine on the activity of the Na<sup>+</sup>/K<sup>+</sup>-ATPase.** Dose and time response studies were conducted. Caco-2 cells were treated with epinephrine for different time intervals (0; 10; 20; 45;75 min) and at different concentrations (0; 0.05; 0.2; 0.5; 0.8 mM). Epinephrine was dissolved in 0.5M ascorbic acid. The positive and negative control groups were incubated with and without ascorbic acid respectively.

**Protein extraction and determination.** At the end of the treatment period, cultured cells were washed twice with PBS, lysed with histidine lysis buffer containing protease inhibitors, scraped, and homogenized at 4°C in a polytron at 22,000rpm. Proteins were quantified colorimetrically at a wavelength of 595nm using the Bradford Biorad assay.

**Na<sup>+</sup>/K<sup>+</sup>-ATPase activity assay.** The activity of the Na<sup>+</sup>/K<sup>+</sup> ATPase was assayed as described by Esmann [14]. Protein concentration of the homogenate was adjusted to 0.5µg/µl using histidine buffer (150mM, pH 7.4). Samples were incubated with 1% saponin, added at a ratio of 1:4(v/v,) and phosphatase inhibitor cocktail for 30 min at room temperature. The final concentration of the cocktail components was: 10.7 mM glycerophosphate, 10.7mM pyrophosphate. Aliquots were then drawn and incubated at 37°C for an additional 30 min in histidine buffer containing NaCl (121.5mM), KCl (19.6 mM), MgCl<sub>2</sub> (3.92 mM), adenosine tri-phosphate (2.94 mM), in presence or absence of ouabain (1.47 mM), a specific inhibitor of the ATPase. At the end of the incubation period, 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 [15].

**Determination of the type of adrenergic receptors involved.** The type of adrenergic receptors mediating the effect of epinephrine on the pump was determined by pre-treating the cells, 20 minutes prior to the addition of epinephrine, with the following antagonists: 0.1 mM yohimbine (α<sub>2</sub> adrenergic antagonist), 50 µM prazosin (α<sub>1</sub> adrenergic antagonist), or 0.03mM propranolol (non-selective β-adrenergic blocker).

Since β adrenergic receptors are coupled to G<sub>s</sub> and the α<sub>2</sub> to G<sub>i</sub>, the effect of dbcAMP (10µM, 20min), a permeable cAMP analogue and RpcAMP (30µM), a PKA inhibitor, was studied. The vehicle was always added to the control in the same amount and for the same time.

**Involvement of PGE<sub>2</sub>.** Previous studies implicated PGE<sub>2</sub> in the epinephrine effect on colonic ion transport. The involvement of PGE<sub>2</sub> was investigated by adding indomethacin (100µM), a COX-inhibitor to the cells, 20 min prior to the addition of epinephrine, and by treating the cells with exogenous PGE<sub>2</sub> (1nM, 20min). The effect of epinephrine was also

investigated in presence of inhibitors of all four different PGE2 receptors, namely: PF-0441848 (1 μM, EP2 antagonist), GW 627368X (10 μM, EP4 antagonist), SC 19220 (100 μM, EP1 antagonist), and L-798106 (1 μM, EP3 antagonist). The inhibitors were added 20 min before epinephrine.

**Involvement of Src.** Adrenergic receptors were shown previously to activate the Src family of tyrosine kinases [16, 17]; hence the involvement of Src in the effect of epinephrine on the ATPase was investigated. The activity of the Na<sup>+</sup>/K<sup>+</sup> ATPase was assayed in Caco-2 cells pre-incubated, 15 min before epinephrine, with PP2 (20 μM), a Src inhibitor. Changes in the expression of phosphorylated Src at Tyr 416 were also studied by western blot analysis.

**Involvement of p38MAPK and ERK.** G protein coupled receptors have been reported to activate mitogen activated protein kinases [18]. To investigate if p38MAPK and ERK have any role to play in the effect of epinephrine on the pump, Caco-2 cells were treated, 15 min before epinephrine with SB 202190 (50 μM), a specific inhibitor of p38MAPK, or with PD98059 (50 μM) a MEK/ERK specific inhibitor.

Changes in the level of phosphorylated ERK and p38MAPK, the active forms of the kinases, were also examined by western blot analysis.

**Locating the different intermediates with respect to PGE2 and with respect to each other.** To determine if each of Src, p38MAPK and ERK is upstream or downstream of PGE2, the cells were treated with epinephrine or PGE2 when each of these kinases was inhibited with respectively PP2, SB 20190 or PD98059.

The effect of epinephrine and PGE2 on the phosphorylation of each of cSrc (Tyr 416), p38MAPK and ERK was also investigated by western blotting.

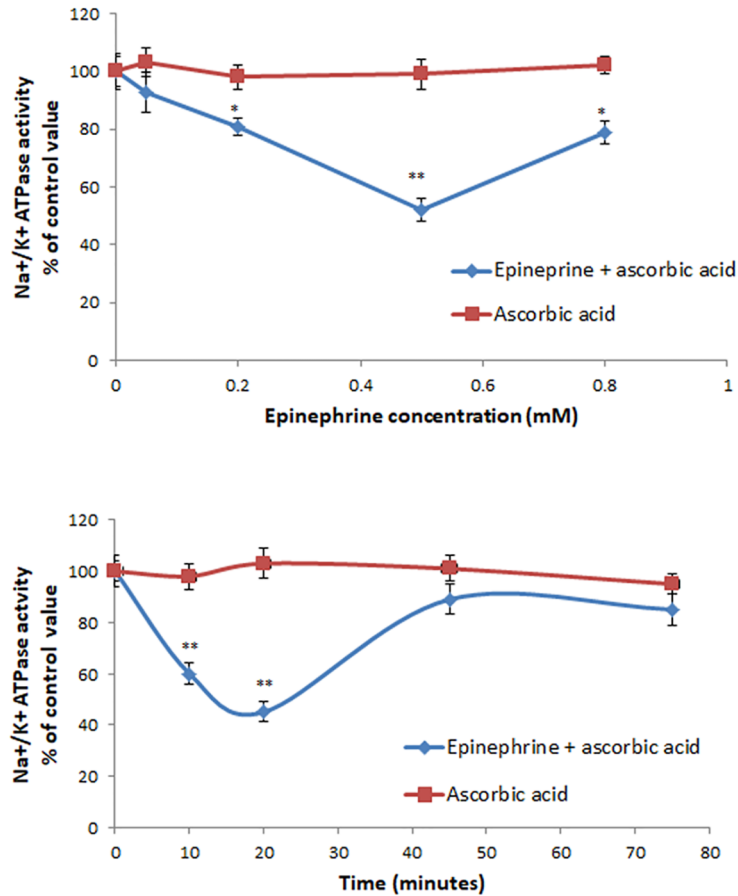
**Western blot analysis.** Treated cells were lysed and homogenized in a polytron (20,000–22,000 rpm) at 4 °C after addition of a cocktail of phosphatase inhibitors. Proteins were quantified using the Bradford method. Equal amounts of proteins (40 μg) were loaded and resolved on 10% SDS polyacrylamide gel and transferred to a nitrocellulose membrane which was then blocked and incubated with a primary ERK1/2, p- ERK1/2, p38α, p -p38α, Src or p-Src antibody, followed by an incubation with a goat anti-rabbit secondary horse raddish peroxidase (HRP) conjugated IgG. The signal was detected by chemiluminescence using Clarity ECL Substrate. The intensity of the signal was detected using a ChemiDoc imager. After probing with an antibody for a phosphorylated kinase, the membrane was sequentially stripped and reprobed with an antibody for the unphosphorylated form of the kinase and then with an antibody for GAPDH. The bands for the phosphorylated kinases were normalized to their total unphosphorylated kinases using Image lab software.

**Statistical analysis.** Results are reported as means ± SEM and are tested for statistical significance by a one-way Analysis of Variance (ANOVA) followed by Tukey-Kramer multiple comparisons test using Instat and Excel Softwares.

## Results

### Dose and time response study on the effect of epinephrine on the Na<sup>+</sup>/K<sup>+</sup>-ATPase

Epinephrine (dissolved in ascorbic acid 0.5M) reduced in a dose and time-dependent manner the activity of the Na<sup>+</sup>/K<sup>+</sup> ATPase in Caco-2 cells. The highest inhibitory effect was observed at 20 min and at a dose of 0.5 mM. Accordingly in all other experiments cell were treated with epinephrine for 20min and at a concentration of 0.5mM. Ascorbic acid alone exerted no significant effect on the activity of the pump (Fig 1).



**Fig 1. Dose (A) and time (A) response study on the effect of epinephrine on the Na<sup>+</sup>/K<sup>+</sup> ATPase activity.** Values are means ±SEM of a minimum of 3 observations. \* Significantly different from the control at P<0.05. \*\* Significantly different from the control at P<0.01.

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### Epinephrine acts via alpha-2 adrenergic receptors

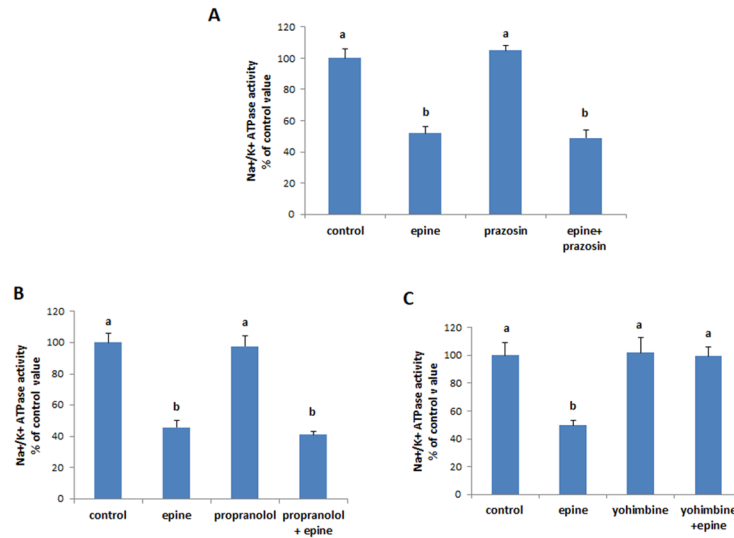
The inhibitory effect of epinephrine on the Na<sup>+</sup>/K<sup>+</sup>-ATPase persisted when the cells were pre-incubated with of 0.03 mM propranolol (non selective β-adrenergic blocker) or 50 μM prazosin (selective α1 antagonist), but was no longer apparent in the presence of 0.1 mM yohimbine (Fig 2), a selective α2- adrenergic antagonist, suggesting that epinephrine exerts its effect by exclusively binding to its α2-adrenergic receptors.

### Epinephrine inhibits PKA

It is well known that α2-adrenergic receptors are coupled to Gi which acts to down-regulate cAMP and inhibit PKA. Treating the cells with RpcAMP (30μM), a cell permeable PKA inhibitor alone, mimicked the inhibitory effect of epinephrine on the Na<sup>+</sup>/K<sup>+</sup>-ATPase, and didn't result in any additive inhibition when added simultaneously with epinephrine (Fig 3A). The cell permeable cAMP analogue dbcAMP, did not have however, any effect on the ATPase (Fig 3B).

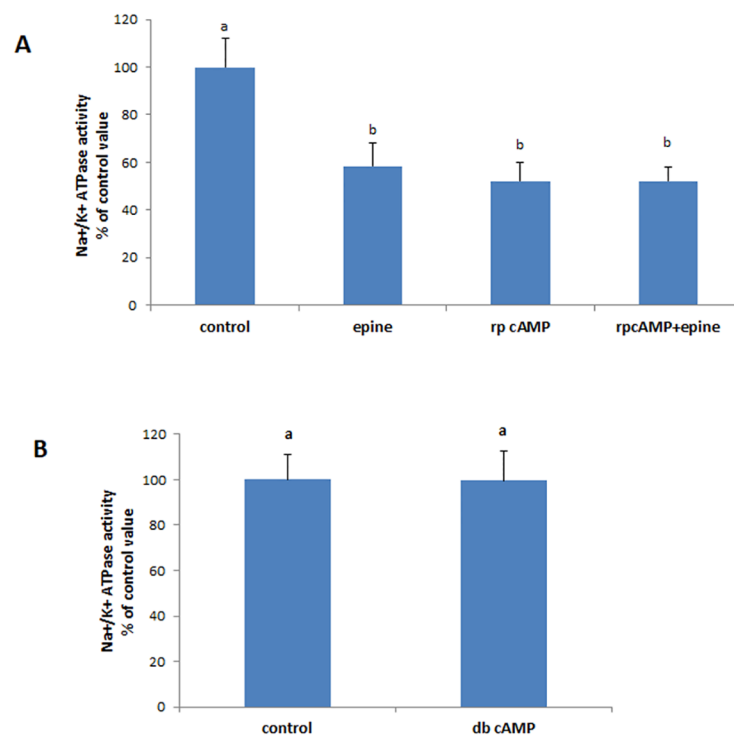
### Determination of the mediators involved

**PGE2.** To test the possibility that epinephrine might be signaling through PGE2, CaCo-2 cells were treated with epinephrine in presence of indomethacin, an inhibitor of COX



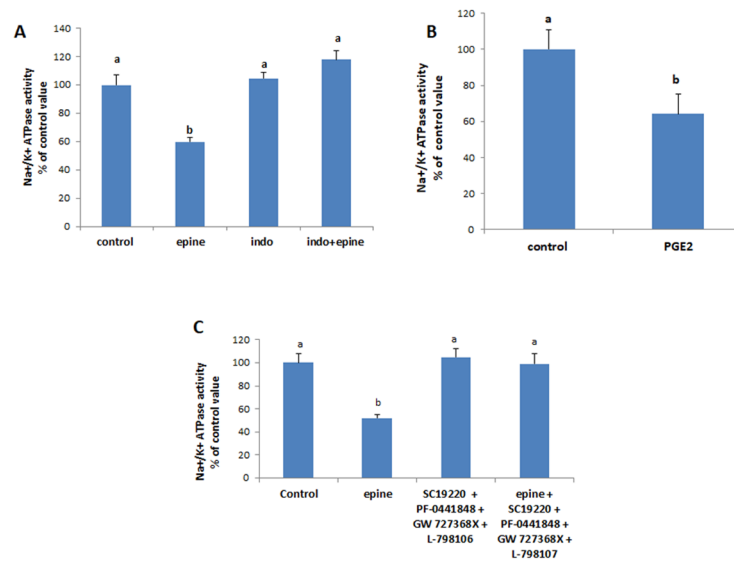
**Fig 2. Epinephrine inhibits the Na<sup>+</sup>/K<sup>+</sup> ATPase by activating  $\alpha$ 2 adrenergic receptors.** Effect of epinephrine (0.5 mM, 20min) on the Na<sup>+</sup>/K<sup>+</sup> ATPase activity in presence of (A) prazosin (50  $\mu$ M), an  $\alpha$ 1 blocker (B) propranolol (0.03M), a  $\beta$  adrenergic blocker, and (C) yohimbine (0.1mM), an  $\alpha$ 2 blocker. Blockers were added 20 min prior to the addition of epinephrine. Values are means  $\pm$ SEM of a minimum of 3 observations. Bars not sharing a common letter are significantly different from each other at P<0.01.

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**Fig 3. (A)RpcAMP (30  $\mu$ M, 20min), an inhibitor of PKA, reduced the Na<sup>+</sup>/K<sup>+</sup> ATPase activity while (B) dbcAMP (10 $\mu$ M, 20min), had no effect.** Values are means  $\pm$ SEM of 3 observations. Bars not sharing a common letter are significantly different from each other at P<0.01.

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**Fig 4. Involvement of PGE2 in epinephrine signaling.** (A) Effect of epinephrine (0.5 mM, 20min) on the activity of the Na<sup>+</sup>/K<sup>+</sup> ATPase in presence of indomethacin (100μM, a COX inhibitor added 20 min before epinephrine); (B) Effect of exogenous PGE2 (1nM,20min) on pump's activity. (C) Effect of epinephrine in presence of inhibitors of all PGE2 receptors. Values are means ± SEM of 4 observations. Bars not sharing the same letter are considered significantly different from each other at p<0.01.

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enzymes. The inhibitor abolished the effect of epinephrine and restored the activity of the Na<sup>+</sup>/K<sup>+</sup>-ATPase back to control levels (Fig 4A). The effect of epinephrine was not manifested when all four PGE2 receptors were blocked with selective antagonists (Fig 4C). Indomethacin alone and the antagonists alone didn't cause any significant change in the activity of the Na<sup>+</sup>/K<sup>+</sup>-ATPase.

To confirm the involvement of PGE2 in the effect of epinephrine, Caco-2 cells were treated with exogenous PGE2. The prostaglandin exerted a similar inhibitory effect to that observed with epinephrine (Fig 4B).

**C-Src.** In presence of the specific inhibitor of c-Src, PP2, the effect of epinephrine disappeared (Fig 5A) but that of PGE2 was maintained (Fig 5B). Similarly, western blot analysis showed a significant increase in the phosphorylation of c-Src(Tyr 416) by epinephrine but not by PGE2(Fig 5C). The expression of un-phosphorylated Src was not affected.

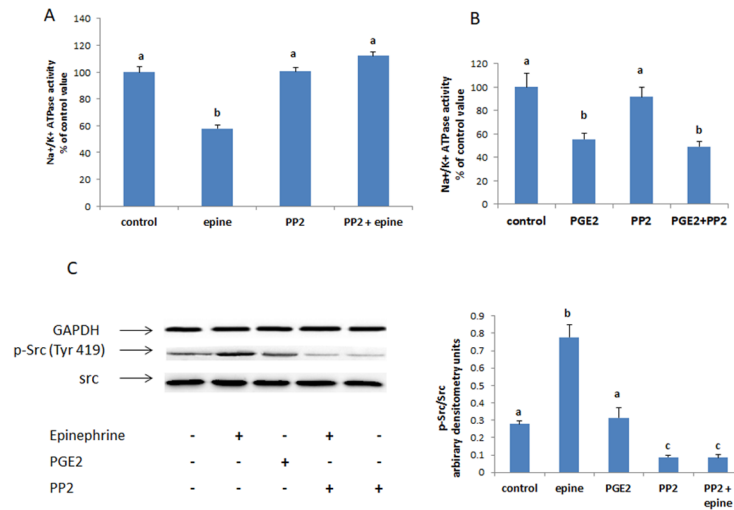
**P38MAPK.** Similarly, inhibition of p38MAPK with SB 202190 obliterated the effect of epinephrine on the ATPase (Fig 6A), but not that of PGE2 (Fig 5B). Western blot analysis showed also a very significant increase in the phosphorylation of p38MAPK that disappeared in presence of PP2 (Fig 5C).

**ERK.** The specific inhibitor of ERK, PD98059, suppressed the effect of epinephrine (Fig 7A) but maintained that of PGE2 (Fig 7B). There was also an increase in the phosphorylation of ERK by epinephrine but not by PGE2 (Fig 7C). This increase disappeared in presence of PP2 or SB202190. Epinephrine did not affect the protein expression of unphosphorylated ERK.

## Discussion

While norepinephrine has been established as a regulator of the Na<sup>+</sup>/K<sup>+</sup>-ATPase in different cells, like brain, kidney and arterial cells [19–21], very few studies investigated the role of its derivative epinephrine on intestinal cells. The scarce literature reports a stimulatory effect of

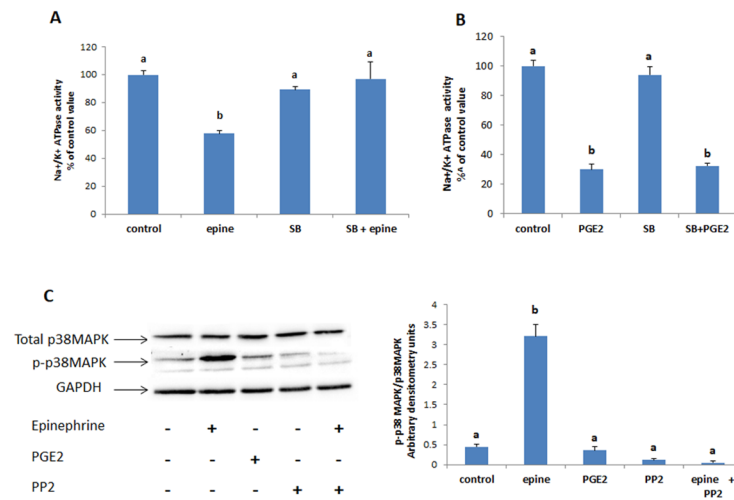




**Fig 5.** (A) Effect of epinephrine (0.5 mM, 20min) and (B) PGE2 (1nM,20min) on the activity of the Na<sup>+</sup>/K<sup>+</sup> ATPase in presence of the Src inhibitor PP2 (20μM, added 15 min before epinephrine or PGE2). Values are means ± SEM of 3 observations. Bars not sharing the same letter are considered significantly different from each other at p<0.01. (C) Epinephrine but not PGE2 increased Src phosphorylation. Values of the normalized data (p-Src/Src) are means ± SEM of 3 observations. Densitometry bars that do not share the same letter are considered significantly different from each other at p<0.01. The blots are representative of an experiment repeated 3 times.

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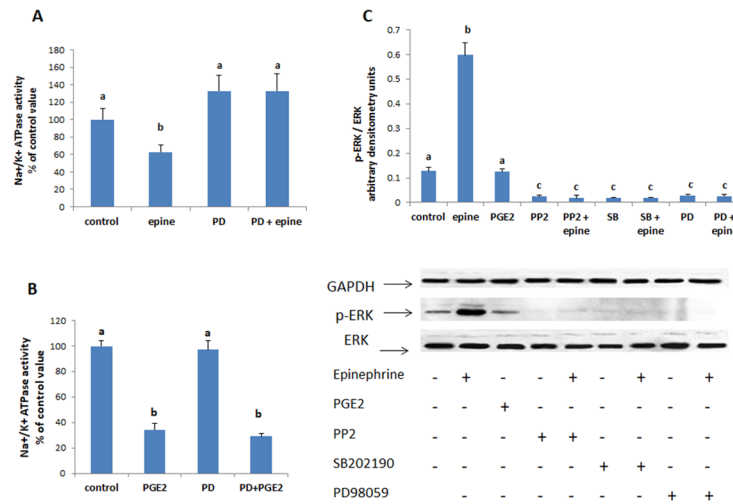
epinephrine on the Na<sup>+</sup>/K<sup>+</sup>-pump in rat jejunal crypt cells [22] and skeletal muscles [23]. Its mechanism of action remains however till now ill-defined. This work studies the effect of epinephrine on the colonic ATPase using Caco-2 cells as a model and tries to elucidate the signaling pathway involved.



**Fig 6.** (A) Effect of epinephrine (0.5 mM, 20min) and (B) PGE2 (1nM,20min) on the activity of the Na<sup>+</sup>/K<sup>+</sup> ATPase when p38MAPK was inhibited with SB202190 (50μM, added 15 min before epinephrine or PGE2). Values are means ± SEM of 3 observations. Bars not sharing the same letter are considered significantly different from each other at p<0.01. (C) Epinephrine increased significantly the phosphorylation of p38MAPK but PGE2 exerted no effect. Values of the normalized data (p38MAPK / p38MAPK) are means ± SEM of 3 observations. Densitometry bars that do not share the same letter are considered significantly different from each other at p<0.01. The blots are representative of an experiment repeated 4 times.

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**Fig 7.** (A) Effect of epinephrine (0.5 mM, 20min) and (B) PGE2 (1nM, 20min) on the activity of the Na<sup>+</sup>/K<sup>+</sup> ATPase when ERK was inhibited with PD98059 (50μM, added 15 min before epinephrine or PGE2). Values are means ± SEM of 3 observations. Bars not sharing the same letter are considered significantly different from each other at p<0.01. (C) Epinephrine but not PGE2 increased significantly the phosphorylation of ERK. Values of the normalized data (p-ERK/ERK) are means ± SEM of 3 observations. Densitometry bars that do not share the same letter are considered significantly different from each other at p<0.01. The blots are representative of an experiment repeated 3 times.

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Treatment of CaCo-2 cells with epinephrine resulted in a decrease in the activity of the Na<sup>+</sup>/K<sup>+</sup>-ATPase that was dose and time dependent with a maximal inhibitory effect at a dose of 0.5mM and an incubation period of 20min. This dose and time dependency may be ascribed to differences in the affinity of the adrenergic receptors, to their desensitization with time, or to the production of signaling intermediates that exert themselves a time dependent effect. The inhibitory effect of epinephrine appears to be mediated via α2-adrenergic receptors exclusively, since it disappeared only in presence of yohimbine, the specific α2 adrenergic blocker, but persisted when epinephrine was simultaneously added with other adrenergic antagonists. Had other receptors been involved, then a partial inhibitory effect would still be observed in presence of yohimbine. Epinephrine's preferential binding to α2-adrenergic receptors can be attributed to the α2-receptors' higher cell surface density, higher affinity to epinephrine, or both, when compared to other types of adrenergic receptors. The differential characteristics of adrenergic receptors in CaCo-2 cells haven't been addressed before; nonetheless, α2- receptors, individually, were shown to be highly expressed in a similar adenocarcinoma cell line, HT-29, and to have the highest affinity to epinephrine among other adrenergic agonists [24].

In accordance with the widely accepted notion that α2-adrenergic receptors are coupled to inhibitory G-proteins (Gi) and act to down-regulate the production of cAMP and consequently PKA activity [25], cells treated with RpcAMP, a PKA inhibitor exhibited a similar decrease in Na<sup>+</sup>/K<sup>+</sup>-ATPase activity as those treated with epinephrine, and the simultaneous addition of epinephrine and RpcAMP didn't result in any additive effect. The data support thus an involvement of Gi in the response to epinephrine. Stimulating PKA with dbcAMP was without any effect on the ATPase.

PKA is known to modulate the Na<sup>+</sup>/K<sup>+</sup> ATPase activity by direct phosphorylation or indirectly through activation of other mediators. PKA phosphorylation at Ser 943 was first identified in the C-terminal of the rat renal Na<sup>+</sup>/K<sup>+</sup>-ATPase α1 subunit [26]. The effects of this phosphorylation in various cell types varies between activation [27], inhibition [28], or no change at all [26]. PKA in this work seems to have a stimulatory effect and is needed for the

basal activity of the ATPase since its inhibition with RpcAMP resulted in a lower ATPase activity.

Alpha-2 adrenergic receptors were reported to signal via PGE2 in various tissues.

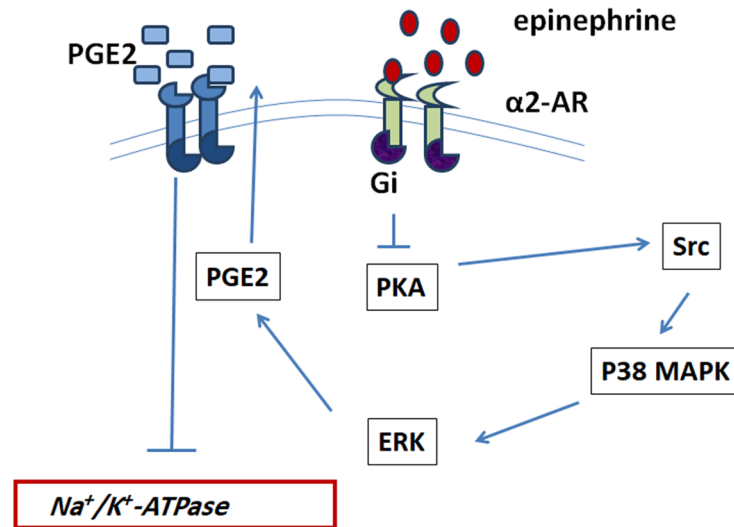
The prostaglandin was found to be responsible for the  $\alpha$ 2 adrenoceptor-dependent urea transport and hyperthermic response in rat IMCD [29] and guinea pigs [30] respectively. Activation of  $\alpha$ 2 adrenergic receptors in cutaneous cells induced also PGE2 release [31].

On the other hand, PGE2 is a recognized regulator of the Na<sup>+</sup>/K<sup>+</sup>-ATPase activity. Its inhibitory effect was observed in several organs including heart [32], liver [33, 34], and kidneys [35]. This work revealed also an inhibitory effect of the prostaglandin in colonic cells, which was behind the epinephrine-induced decrease in the Na<sup>+</sup>/K<sup>+</sup> ATPase activity, since the effect of the hormone on the ATPase was suppressed when the synthesis of PGE2 was blocked with indomethacin.

The inhibitory effect of epinephrine on the Na<sup>+</sup>/K<sup>+</sup> ATPase was also abrogated in presence of PP2, an inhibitor of the cytoplasmic tyrosine kinase Src, but that of PGE2 was maintained, implying that Src is involved in epinephrine's signaling and is upstream of PGE2. Src activity is regulated by phosphorylation. While phosphorylation at tyrosine Y527 inhibits the kinase by stabilizing a confirmation that prevents interaction with the substrate, phosphorylation at tyrosine Y416 increases the activity of Src by stabilizing the activation loop [36]. Western blot analysis showed a greater epinephrine but not PGE2-induced phosphorylation of Src (Tyr 416), confirming the involvement and position of Src in the signaling pathway. Such an involvement came as no surprise since G protein coupled receptors in general [37] and alpha-2 adrenergic receptors in particular, are known to increase the activity of Src kinase in a pertussis toxin sensitive manner, inferring that this increase is mediated via a Gi protein [38]. Src is usually maintained in an inactive state by phosphorylation of the C-terminal regulatory tyrosine by C-terminal Src kinase (Csk). Abrahamsen et al. [39] demonstrated that PKA increases the activity of Csk and consequently represses Src. The stimulation of Src observed in this work may be due the Gi- induced inhibition of PKA which in turn decreases the activity of Csk and relieves Src of its inhibitory effect.

We identified in addition, p38MAPK as another mediator of epinephrine signaling. In presence of SB202190, a specific inhibitor of the kinase, the effect of epinephrine on the Na<sup>+</sup>/K<sup>+</sup> pump did not appear, while that of PGE2 was still observed, suggesting that p38MAPK is upstream of PGE2. This conclusion was supported by the western blots which showed a very significant increase in the phosphorylation of p38MAPK by epinephrine. This increase was not manifested when Src kinase was inhibited with PP2, indicating that Src is upstream of p38MAPK. The findings are in line with those of Thobe et al. [40] who demonstrated in mice Kupffer cells, an activation of p38MAPK in response to hypoxia that was induced by Src activation. Similarly TGF $\beta$ 1 was shown by Pechkovsky et al. [41] to increase  $\alpha$ v $\beta$ 3 integrin expression in human lung fibroblasts through a c-Src dependent activation of p38MAPK. Activation of p38 by Src was also shown to be required for EGF-stimulated intestinal epithelial monolayer restitution [42].

Another MAPK found to be involved in the inhibitory effect of epinephrine is ERK: The activity of the ATPase was restored back to control values in cells treated with epinephrine in presence of PD98059, an inhibitor of MEK/ERK. The inhibitory effect of PGE2 was, however, not affected by ERK inhibition, inferring that ERK works upstream of PGE2. Western blot analysis showed also a significant increase in ERK phosphorylation upon treatment with epinephrine but not upon treatment with PGE2. The epinephrine—induced increase in p-ERK expression did not appear when Src and p38 MAPK were inhibited respectively with PP2 and SB202190, implying that ERK is downstream of p38MAPK which is downstream of Src. Whether p38MAPK activates ERK by cross-talking directly or indirectly with one or more of the components of the ERK module is not clear and needs further investigation. Up to our



**Fig 8. Proposed signaling pathway of epinephrine’s action on the Na<sup>+</sup>/K<sup>+</sup> ATPase.**

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knowledge, no previous work reported a p38MAPK dependent activation of ERK. The interactions reported in the literature between the two kinases are of a negative nature [43] and mediated via the protein phosphatase 2A [44].

Sine ERK is upstream of PGE2, it may play a role in its production. PGE2 synthesis is catalyzed by the COX enzymes, COX-2 being the inducible isoform. The activation of COX-2 by ERK has been frequently reported. Chae et al. [45] showed that inhibition of ERK pathway blocked the TNF-α induced PGE2 release. ERK was shown also to mediate the increase in COX-2 expression induced by thrombin in human lung fibroblasts [46] as well as the PGE2 release responsible for neuronal death in TDP-43-depleted microglia [47] and the salicylate induced increase in COX-2 expression in osteoblasts [45].

PGE2 appears to act as a negative modulator of the pump in a variety of tissues. An increase in PGE2, associated with a decrease in cAMP, mediated angiotension II inhibitory effects on Na<sup>+</sup>/K<sup>+</sup>-ATPase and water absorption in rat jejunum [48]. PGE2 was also shown to reduce Na<sup>+</sup>/K<sup>+</sup>-ATPase protein expression in LLCPK1 [49], cardiomyocytes [32], and HepG2 cells [33]. Incubation of rat hippocampus, both *in vivo* and *in vitro*, with PGE2 for 30 min led to a dose dependent decrease in the Na<sup>+</sup>/K<sup>+</sup>-ATPase activity attributed to the PKA and PKC-dependent Ser943 phosphorylation of the α subunit [50].

It can be concluded that epinephrine inhibits the Na<sup>+</sup>/K<sup>+</sup> ATPase in Caco-2 cells via α2 adrenergic receptors leading to the sequential activation of Src, p38MAP, ERK, COX-2 and eventually PGE2 release. The prostaglandin reduces the activity of the ATPase through another signaling pathway that needs to be explored.

The pathway involved in the effect of epinephrine on the Na<sup>+</sup>/K<sup>+</sup> ATPase is represented in Fig 8.

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