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RESEARCH PAPER

# The endocannabinoid system in renal cells: regulation of $Na^+$ transport by $CB_1$ receptors through distinct cell signalling pathways

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## BACKGROUND AND PURPOSE

The function of the endocannabinoid system (ECS) in renal tissue is not completely understood. Kidney function is closely related to ion reabsorption in the proximal tubule, the nephron segment responsible for the re-absorption of 70–80% of the filtrate. We studied the effect of compounds modulating the activity of cannabinoid (CB) receptors on the active re-absorption of $Na^+$ in LLC-PK1 cells.

## EXPERIMENTAL APPROACH

Changes in $Na^+/K^+$-ATPase activity were assessed after treatment with WIN55,212-2 (WIN), a non-selective lipid agonist, and haemopressin (HP), an inverse peptide agonist at $CB_1$ receptors. Pharmacological tools were used to investigate the signalling pathways involved in the modulation of $Na^+$ transport.

## KEY RESULTS

In addition to $CB_1$ and $CB_2$ receptors and TRPV1 channels, the mRNAs encoding for enzymes of the ECS were also expressed in LLC-PK1. WIN ($10^{-7}$ M) and HP ($10^{-6}$ M) altered $Na^+$ re-absorption in LLC-PK1 in a dual manner. They both acutely (after 1 min) increased $Na^+/K^+$-ATPase activity in a TRPV1 antagonist-sensitive way. WIN's stimulating effect persisted for 30 min, and this effect was partially blocked by a $CB_1$ antagonist or a PKC inhibitor. In contrast, HP inhibited $Na^+/K^+$-ATPase after 30 min incubation, and this effect was attenuated by a $CB_1$ antagonist or a PKA inhibitor.

## CONCLUSION AND IMPLICATIONS

The ECS is expressed in LLC-PK1 cells. Both $CB_1$ receptors and TRPV1 channels regulate $Na^+/K^+$-ATPase activity in these cells, and are modulated by lipid and peptide $CB_1$ receptor ligands, which act via different signalling pathways.

### Abbreviations

2-AG, 2-arachidonoylglycerol; AEA, anandamide; CPZ, capsazepine; DGL, diacylglycerol lipase; ECS, endocannabinoid system; FAAH, fatty acid amide hydrolase; HP, haemopressin ($CB_1$ inverse agonist); LLC-PK1 cells, immortalized epithelial cells derived from pig kidney proximal tubule; MGL, monoacylglycerol lipase; NAPE-PLD, *N*-acylphosphatidylethanolamine-PLD; pERK1/2, phospho-ERK1/2; PTX, pertussis toxin; RVD-HP, RVD-haemopressin (haemoglobin-derived peptides; $CB_1$ agonists); VD-HP, VD-haemopressin ($CB_1$ agonist); WIN, WIN55,212-2

## Tables of Links

| TARGETS | |
|---|---|
| **GPCRs**[a] | **Enzymes**[d] |
| $CB_1$ receptor | DGL |
| $CB_2$ receptor | ERK1 |
| **Ion channels**[b] | ERK2 |
| TRPV1 | FAAH |
| **Transporters**[c] | PKA |
| $Na^+/K^+$-ATPase | PKC |

| LIGANDS | |
|---|---|
| 2-AG | Capsazepine |
| AM251 | H89 |
| Anandamide | IBMX |
| Calphostin C | Ouabain |
| cAMP | WIN55,212-2 |

These Tables list key protein targets and ligands in this article which are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Pawson *et al.*, 2014) and are permanently archived in the Concise Guide to PHARMACOLOGY 2013/14 ([a,b,c,d]Alexander *et al.*, 2013a,b,c,d).

## Introduction

In mammals, the endocannabinoid system (ECS) is classically represented by (i) several endogenous lipids, such as anandamide (AEA) and 2-arachidonoylglycerol (2-AG); (ii) cannabinoid receptors; and (iii) enzymes responsible for the synthesis and degradation of these lipids (Howlett, 2002). $CB_1$ and $CB_2$ receptors are members of the GPCR family, both of which are primarily coupled to the $G_i$ protein leading to adenylate cyclase inhibition, thereby reducing cAMP production (Devane *et al.*, 1988; Howlett *et al.*, 1990). AEA can act on other receptors, such as TRPV1, an ion channel of the endovanilloid system (Zygmunt *et al.*, 1999). Despite the well-known lipidic endogenous cannabinoids, Heimann and co-workers (2007) showed that haemopressin (HP), a nonapeptide produced by the *in vitro* cleavage of the α1chain of haemoglobin, acts as an inverse agonist of the $CB_1$ receptor. *In vivo*, the major endogenous peptides that interact with the ECS are RVD-HP (RVDPVNFKLLSH) and VD-HP (VDPVNFKLLSH), which behave as agonists (Gomes *et al.*, 2010). Although the ECS is abundantly expressed in the nervous system, it is also present in the immune, reproductive and renal systems (Devane *et al.*, 1988; Howlett *et al.*, 1990; Matsuda *et al.*, 1990; Di Marzo *et al.*, 1998). In the latter system, the ECS maintains renal function by its ability to promote the relaxation of the kidney endothelium (Deutsch *et al.*, 1997). Cannabinoids also dilate afferent and efferent arteries, decrease the glomerular filtration rate (Koura *et al.*, 2004), reduce blood pressure via the $CB_1$ receptor, and increase urine volume due to their interaction with TRPV1 receptors (Li and Wang, 2006). However, the mechanisms related to its effects on renal tissue are not fully understood.

We investigated the presence of the ECS in LLC-PK1 (immortalized epithelial cells derived from pig kidney proximal tubule), a renal proximal tubule cell line, by measuring the expression of $CB_1$ and $CB_2$ receptors and the main enzymes involved in the synthesis and degradation of AEA and 2-AG [*N*-acylphosphatidylethanolamine-PLD (NAPE-PLD), fatty acid amide hydrolase (FAAH), diacylglycerol lipase (DGL) and monoacylglycerol lipase (MGL)], by biochemical, pharmacological and morphological analysis. We also hypothesized that lipidic and peptide cannabinoids might regulate $Na^+/K^+$-ATPase activity, a $Na^+$ pump present in the renal proximal tubular epithelium that is important for maintaining the volume and composition of the extracellular fluid through different signalling pathways.

## Methods

### *Nomenclature*

All the drug/molecular target nomenclature conforms to BJP's Concise Guide to Pharmacology (Alexander *et al.*, 2013).

### *Kidney epithelial cell culture*

LLC-PK1 cells (immortalized epithelial cells derived from pig kidney proximal tubule; ATCC – LLC-PK1: ATCC® CL-101™) were grown in DMEM supplemented with 10% FBS for 48 h at 37°C in an air with 5% $CO_2$. Experiments involved cultures that were 90% confluent; the cells had adhered to the flask and their polarity was preserved, an important characteristic of this cell line. After treatment with the various drugs, the cells were washed with PBS, scraped from the flasks and homogenized. Total protein concentration was measured by the method of Lowry *et al.* (1951).

### *Quantitative real-time qRT-PCR*

Total RNA was isolated from LLC-PK1 cells with TRIzol-Reagent (Sigma-Aldrich, Milan, Italy), reacted with DNase-I

**Table 1**
Primers used for qRT-PCR in LLC-PK1 cells

| | Forward primer | Reverse primer |
|---|---|---|
| NAPE-PLD | 5′ACCGGCCTCTGAGAAAATGG3′ | 5′AGGGTTAACTGGGGAGACCT3′ |
| DGL-α | 5′GAAACCAAACACGCCTCCAC3′ | 5′CAACCCAGCAGCAAAGGAAC3′ |
| DGL-β | 5′TTTGTAATCCCGGACCACGG3′ | 5′ATTCTCGTTTCCCACACGCT3′ |
| FAAH | 5′TGCCACCGTGCAAGAAAATG3′ | 5′CCACTGCCCTAACAACGACT3′ |
| MGL | 5′CACTTCTCCGGCATGGTTCT3′ | 5′CGTGAAACGGCGTTGAGC3′ |
| $CB_1$ | 5′CGCCAATGACATTCAGGGAAG3′ | 5′CGGGAATGGAGGATAACGCA3′ |
| $CB_2$ | 5′TTTATAGCCTGGCCTCCCCT3′ | 5′TTTTCCCGTCTGCCTCTGTC3′ |
| TRPV1 | 5′TAAGACCAGCTCCAGCCAAG3′ | 5′AACTCGCTGTCCACCAGATG3′ |
| S16 | 5′TCATCAAAGTGAACGGGCGA3′ | 5′GACGAGGATCAGCTACCAGC3′ |

(1 U·mL$^{-1}$; Sigma-Aldrich) for 15 min at room temperature, and followed by spectrophotometric quantification. The final preparation of RNA was essentially DNA-and protein-free when the ratio of readings at 260 and 280 nm were ≥1.7. Isolated mRNA was reverse-transcribed with iScript kit Reverse Transcriptase (Bio-Rad, Milan, Italy). Quantitative real-time PCR was carried out in a CFX384 real-time PCR detection system (Bio-Rad, Milan, Italy), with specific primers for the main enzymes involved in the synthesis and degradation of AEA or 2-AG (Table 1); SYBR Green was used as the detection agent (Bio-Rad, Segrate MI, Italy). Samples were amplified simultaneously in quadruplicate in one-assay run with a non-template blank for each primer pair as a control for contamination or the formation of primer-dimers; the ct (cycle threshold) value for each experimental group was determined. The housekeeping gene, ribosomal protein S16, an internal control to normalize the ct values using the formula $2^{-\Delta Ct}$. Differences in mRNA content between groups are as expressed as $2^{-\Delta\Delta ct}$ (Iannotti *et al.*, 2013).

## *Electrophoresis and immunodetection*

Proteins in the cell homogenate were separated by 10% SDS-PAGE and transferred to nitrocellulose membranes (Laemmli, 1970). Immunodetection was performed by incubating the samples with a specific primary antibody (anti-ERK1/2 or anti-pERK1/2, 1:1000 dilutions) from Cell Signaling, for at least 24 h, whereas incubation with the secondary antibody (Peroxidase labelled ECL™; GE Healthcare, Buckinghamshire, UK) was for only 2 h. The proteins of interest were finally detected using the ECL system and a Hyperfilm (Hyperfilm-ECL – GE Healthcare).

## *Immunofluorescence*

LLC-PK1 cells were grown on 13 mm coverslips for 48 h, after which they were washed with PBS (pH 7.4) and fixed for 15 min in paraformaldehyde (4%) in 0.1 M PBS. The coverslips were washed three times with PBS and incubated for 15 min with 0.5%Triton X-100 in PBS (v/v) before the samples were incubated for 2 h at room temperature with a blocking buffer (3% donkey serum in PBS). This buffer was removed and the primary antibodies of interest added [for $Na^+/K^+$-ATPase (1:100), $CB_1$ (1:100), and $CB_2$ (1:100) receptors and TRPV1(1:100)] before the samples were incubated for 24 h at 4°C. The coverslips were washed with PBS and fluorochrome-conjugated secondary antibody added for 3 h at room temperature in the dark. Finally, coverslips were examined by use of a fluorescence microscope (ApoTome 2® – Zeiss, Oberkochen, Germany).

## *$Na^+/K^+$-ATPase activity*

$Na^+/K^+$-ATPase activity was measured colorimetrically by quantifying the difference in inorganic phosphate ($P_i$) released from ATP (Taussky and Shorr, 1953) in the absence and presence of ouabain (2 mM), a specific inhibitor of $Na^+/K^+$-ATPase. LLC-PK1 homogenates were prepared after treatment with the drugs in each experimental set.

## *cAMP quantification assay*

cAMP content was measured by the competitive binding assay (De Mello, 1978). To promote the accumulation of cAMP, the LLC-PK1 cells were treated with IBMX (500 µM), an inhibitor of PDEs, for 15 min before the drugs were added. The reaction was stopped by the addition of TCA 100% (w/v) and the supernatant was transferred to plastic tubes for storage at –20°C for 24 h. After centrifugation (1000× *g* for 30 min), the supernatant was eluted through an ion-exchange resin column (Dowex AG50W-X8 200–400 mesh; Sigma-Aldrich, St. Louis, MO, USA) to remove TCA and nucleotides. Samples containing cAMP were incubated in the presence of the regulatory subunit of PKA and a trace amount of [$^3$H]-cAMP to compete for the PKA site, using 50 mM acetate buffer at pH 4.0 for 90 min at 4°C. The reaction was stopped by adding 200 mM phosphate buffer at pH 6.0. The samples were filtered through Millipore (Merck, Darmstadt, Germany) filters (0.22 µm) and the bound radioactivity measured in a liquid scintillation analyser (Tri-Carb 2810 TR, Perkin Elmer, Hopkinton, MA, USA).

## *Statistical analysis*

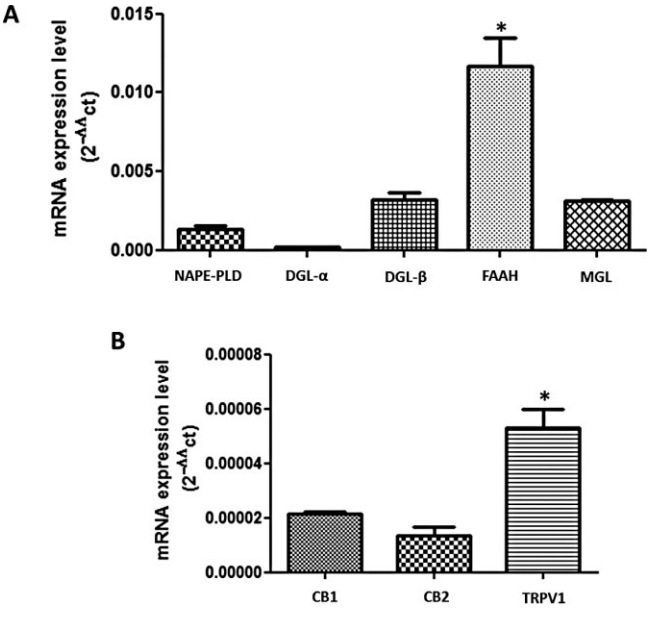
Assays were often done in quadruplicate to ensure reliability of individual independent values. Results are presented as mean ± SEM. Graphpad Prism®5 (La Jolla, CA, USA) software was used for analysing the results by one-way ANOVA, followed by

Tukey's post-test. $P < 0.05$ was taken as the significance level, and the number of experiments is shown in each figure legend.

*Materials*. All the materials used were of the highest analytical grade. Antibodies against cannabinoid receptors, $CB_1$ and $CB_2$ were from Proteimax (São Paulo, Brazil), $Na^+/K^+$-ATPase from Sigma-Aldrich and TRPV1 from Santa Cruz (Dallas, TX, USA). Anti-ERK1/2 and phospho-ERK1/2 (pERK1/2) were from Cell Signaling (Beverly, MA, USA). WIN55,212-2 and AM251 were purchased from Cayman Chemical (Ann Arbor, MI, USA). HP, RVD-HP and VD-HP (RVD-HP and VD-HP) were obtained from Proteimax. H-89, Calphostin C and pertussis toxin (PTX) were from Sigma-Aldrich. Reagents for RT-PCR were from BioRad (Hercules, CA, USA), and primers were from Eurofins MWG (Ebersberg, Germany) operon (Table 1).

## Results

The ability of LLC-PK1 cells to express the main components of the ECS was analysed first by qRT-PCR. Figure 1A shows that the main enzymes of the ECS, for AEA (NAPE-PLD) and 2-AG (DGLα and DGLβ) biosynthesis, and AEA (FAAH) and 2-AG (MGL) degradation are expressed in LLC-PK1 cells. Also, cannabinoid receptors ($CB_1$, $CB_2$) and TRPV1 were shown by qRT-PCR (Figure 1B) and immunofluorescence (Figure 2). The expression of $Na^+/K^+$-ATPase (red) was also obvious, and it was exclusively present in the basolateral membranes of these cells (third column in Figure 2). The expression of $CB_1$ and $CB_2$ receptors and TRPV1 appear as green, in rows A, B and C of Figure 2 respectively. The merged images show that the location of the $CB_1$, $CB_2$ and TRPV1 receptors is different from that of $Na^+/K^+$-ATPase, suggesting an apical distribution (fourth column, Figure 2). Negative controls for each marker are depicted in the first column (Figure 2).




## Figure 1

qRT-PCR for the main enzymes and receptors of ECS in LLC-PK1 cells. (A) Detection of mRNA for the enzymes responsible for AEA (NAPE-PLD) and 2A-G (DGL-α and β) synthesis, respectively. The main enzymes for the degradation of AEA and 2-AG, FAAH and MGL, respectively, were also expressed. (B) LLC-PK1 cells also possess the mRNA for $CB_1$, $CB_2$ and TRPV1 receptors. Data are means ± SEM of $n = 3$; *Indicates different from control ($P < 0.05$). We also calculated the Power Test (Error Type II β), which was 99.9%.

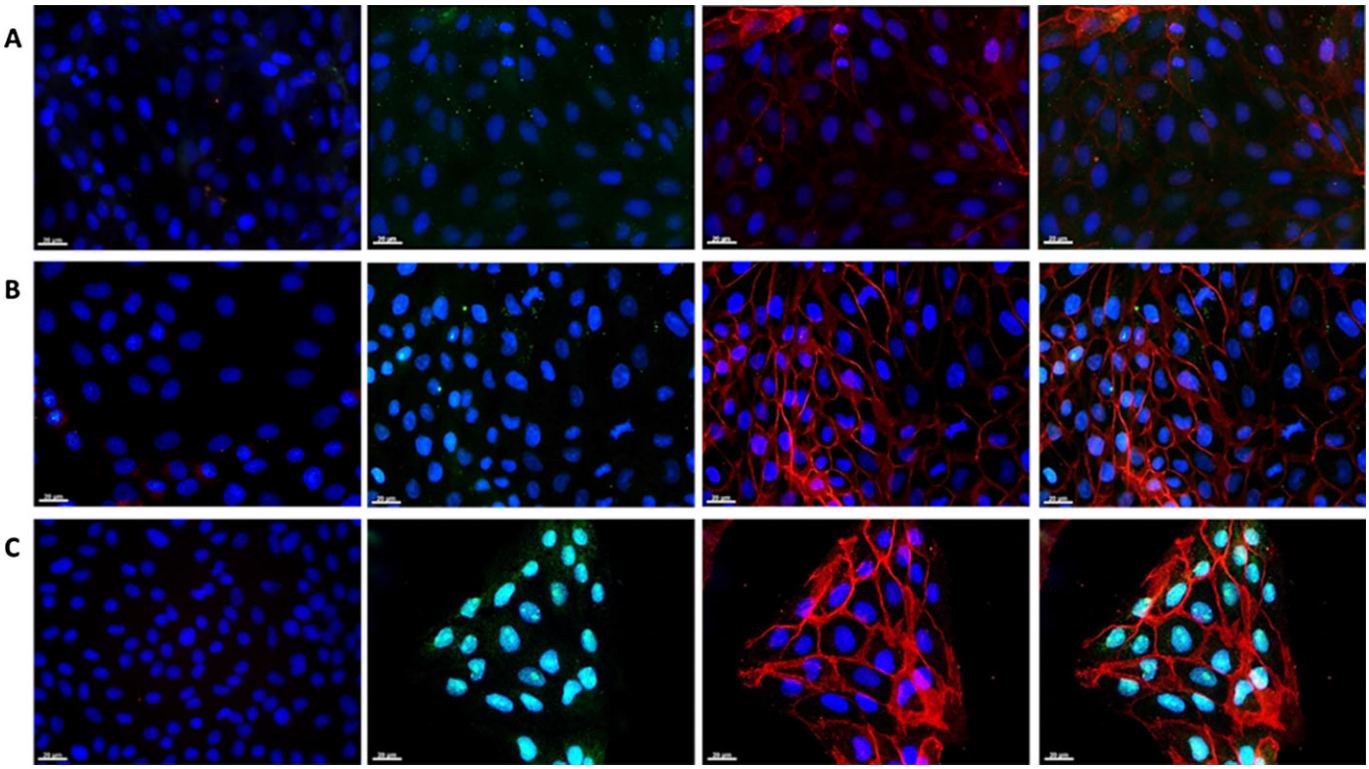
To test whether the $Na^+/K^+$-ATPase activity is modulated by cannabinoids, LLC-PK1 cells were treated with the non-selective $CB_1/CB_2$ agonist, WIN at different concentrations ($10^{-9}$, $10^{-8}$ and $10^{-7}$ M). Figure 3A shows that only $10^{-7}$ M WIN lead to a significant increase in $Na^+/K^+$-ATPase activity after 30 min. Figure 3B shows that this effect initially occurs after 1 min and is sustained until 30 min of treatment. We also tested different concentrations ($10^{-7}$ and $10^{-6}$ M) of HP, an inverse agonist of the $CB_1$ receptor, and Figure 4A clearly shows that after 30 min of incubation both concentrations lead to a significant inhibition of $Na^+/K^+$-ATPase activity. Surprisingly, $10^{-6}$ M HP displayed a biphasic effect (Figure 4B), leading to a significant increase in the $Na^+/K^+$-ATPase activity after 1 min, whereas it acted as a potent inhibitor of the pump after 15 min; this effect was sustained for 30 min. Thus HP behaved in an opposite manner to that observed for WIN at longer incubation times.

Since WIN is a non-selective $CB_1/CB_2$ receptor agonist, we tested whether the addition of AM251, a selective $CB_1$ receptor antagonist, prevented the activation of $Na^+/K^+$-ATPase induced by WIN. AM251 at $10^{-7}$ M completely abolished the effect of WIN on $Na^+/K^+$-ATPase activity after 30 min, but had no effect at 1 min (Figure 5A). Similarly, $10^{-7}$ M AM251 had no effect on $Na^+/K^+$-ATPase activity stimulated by HP when pre-incubated for 1 min, but abolished the inhibitory effect of WIN observed after 30 min incubation (Figure 5B). These results suggest that $Na^+/K^+$-ATPase can be modulated by, at least, two different regulatory mechanisms induced by cannabinoids: (i) a fast one (1 min) that is independent of $CB_1$ receptors; and (ii) a slow one (30 min) that is dependent on $CB_1$ receptors.

Some cannabinoid drugs can trigger TRPV1 receptors in different cell lines (Jeske *et al.*, 2006; Patwardhan *et al.*, 2006). To explore a possible involvement of TRPV1 receptors in the modulation of $Na^+/K^+$-ATPase activity induced by cannabinoids, we first confirmed by immunofluorescence that the LLC-PK1 cells express TRPV1 (Figure 2C). Pre-incubation (10 min) of LLC-PK1 cells with $10^{-7}$ M capsazepine (CPZ), a TRPV1 antagonist, completely abolished the fast stimulating effect of either WIN or HP on $Na^+/K^+$-ATPase activity (Figure 6).

To show that endogenous cannabinoid peptides are also able to trigger $CB_1$ receptors in LLC-PK1 cells, we used RVD-HP and VD-HP. Figure 7 shows that both increased $Na^+/K^+$-ATPase activity.

The next step was to investigate the cell signalling pathway downstream of the activation of $CB_1$ receptors. LLC-PK1 cells were pretreated with 100 ng·mL$^{-1}$ PTX, an inhibitor of the $\alpha_i$ subunit of G protein (Skorecki *et al.*, 1987) and we did not observe any alteration in the regulatory effects of WIN or HP on the $Na^+/K^+$-ATPase activity (Figure 8A), which allowed us to rule out the participation of a $G_i$ in the response to $CB_1$ receptor activation. Furthermore, as the activation of $CB_1$ receptors can both decrease or, less frequently, increase adenylate cyclase activity (Glass and Felder, 1997), we decided to measure the cAMP levels in LLC-PK1 cells upon




## Figure 2

Immunofluorescence of $CB_1$ and $CB_2$ receptors, and TRPV1 channels. In all the panels, cell nuclei are marked in blue (DAPI), cannabinoid receptors are in green (Alexa 488) and $Na^+/K^+$-ATPase in red (Alexa 594). Rows are as follows (A) $CB_1$, (B) $CB_2$ and (C) TRPV1. Columns are as follows: first column is the negative control (no primary antibody incubation); the second shows the expression of each studied receptor; the third presents the $Na^+/K^+$-ATPase distribution; and the fourth is the merged image of each receptor (green) and $Na^+/K^+$-ATPase (red). Representative images from three different immunofluorescence assays for each receptor. Magnification 40×.

stimulation by the cannabinoids. We observed that the addition of $10^{-7}$ M WIN for 30 min had no effect on the cytosolic cAMP levels. However, $10^{-6}$ M HP significant increased the cAMP levels after 30 min, an effect that was completely abolished by the pre-incubation of the cells with AM251 (Figure 8B). Due to the changes in cAMP levels, we also evaluated the possible role of PKA. After a 10 min pretreatment with $10^{-5}$ M H89, a permeable PKA inhibitor (Chijiwa *et al.*, 1990), the stimulating effect induced by $10^{-7}$ M WIN on $Na^+/K^+$-ATPase activity was not impaired (Figure 9A). However, inhibition of PKA abolished the inhibitory effect induced by $10^{-6}$ M HP (Figure 9A).

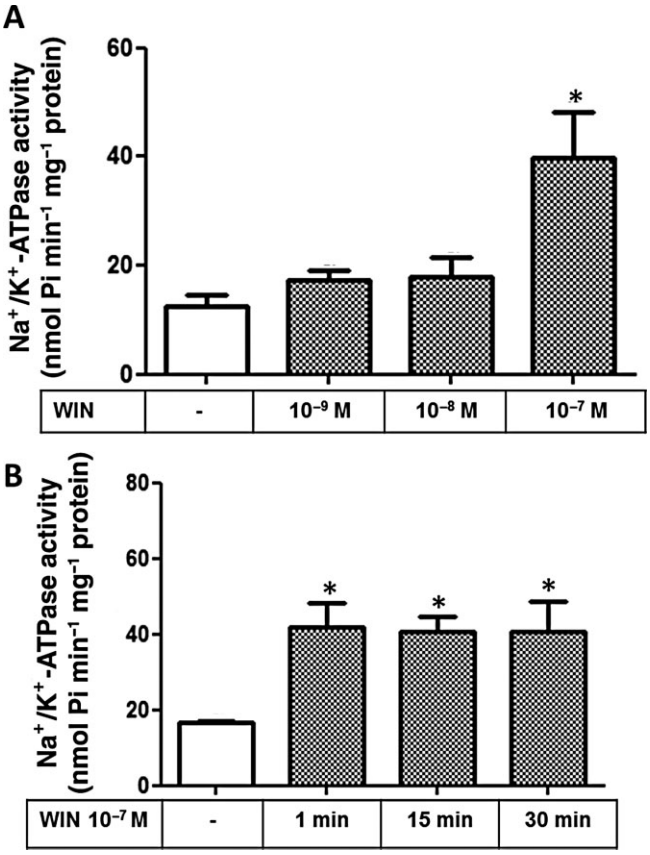
PKC-dependent events are very important and frequent in LLC-PK1 cells (Peruchetti *et al.*, 2011). Therefore, we decided to investigate whether PKC was involved in the modulation of $Na^+/K^+$-ATPase by WIN or HP. Pre-incubation of the cells with $10^{-8}$ M calphostin C, a selective PKC inhibitor for classic and novel PKC isoforms at this concentration (Kinoshita *et al.*, 1990), completely inhibited the stimulating effect of WIN on the $Na^+/K^+$-ATPase activity for 30 min, whereas it had no effect on the inhibitory action of HP (Figure 9B).

Finally, the activation of cannabinoid receptors is commonly associated with the activation of different signalling cascades that culminate in the activation of protein kinases involved in cell proliferation, survival and apoptosis (Bouaboula *et al.*, 1995). In this scenario, MAPKs, such as the ERKs could be involved in the effects of cannabinoids in LLC-PK1 cells. Figure 10 shows that $10^{-6}$ M HP decreased ERK1/2 phosphorylation after 30 min, an effect that was abolished by the pretreatment with AM251. However, WIN had no effect on the phosphorylation of ERK1/2.

## Discussion

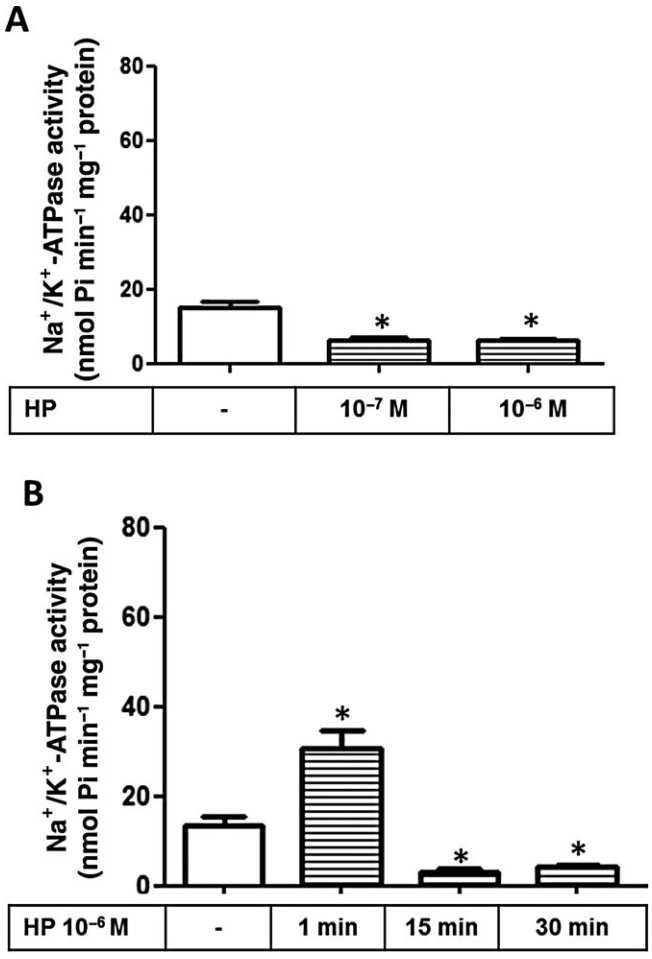
Since it was first described in the CNS, the ECS is now thought to be a regulator of different physiological mechanisms. More information is now available about this signalling system, but, as yet, there is little evidence for its role outside the CNS. In the present study, we have shown that the ECS can affect renal $Na^+$ reabsorption by directly stimulating $Na^+/K^+$-ATPase in LLC-PK1 cells (kidney epithelia).

$Na^+/K^+$-ATPase, located in the basolateral membrane of tubular cells, is the main system responsible for $Na^+$ reabsorption and the generation of a $Na^+$ gradient across the epithelium, and is an important target for hormones, autacoids and bioactive molecules in general within the nephron segments (Schwartz *et al.*, 1974). Recently, cannabinoids were shown to be modulators of $Na^+/K^+$-ATPase in different tissues; Araya and co-workers demonstrated that they can modulate synaptosomal $Na^+/K^+$-ATPase via stimulation of $CB_1$ receptors (Araya *et al.*, 2007).




## Figure 3

Effect of WIN on the $Na^+/K^+$-ATPase activity in LLC-PK1 cells. The $Na^+/K^+$-ATPase activity was determined according to Methods. (A) The different concentrations of WIN used are depicted below the bars. (B) time-course of modulation of $Na^+/K^+$-ATPase activity of LLC-PK1 cells treated with $10^{-7}$ M WIN. Data are means ± SEM from six different experiments ($n$ = 6); *Indicates different from control ($P < 0.05$).

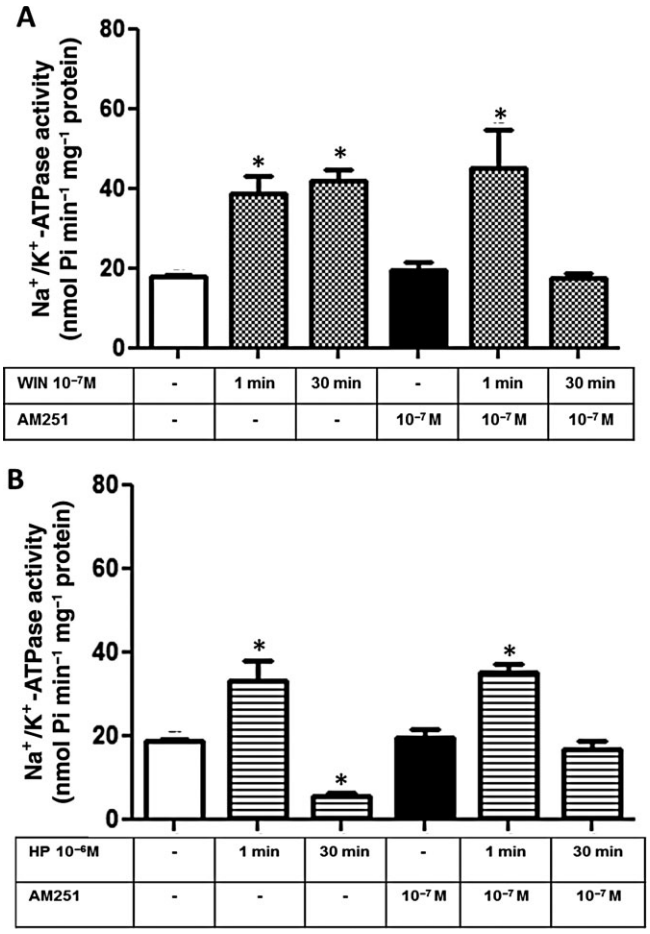



## Figure 4

Effect of HP on $Na^+/K^+$-ATPase activity in LLC-PK1 cells. The $Na^+/K^+$-ATPase activity was determined according to Methods. (A) Cells were incubated for 30 min with two different concentrations of HP ($10^{-7}$ M and $10^{-6}$ M), a $CB_1$ inverse agonist, as depicted. Data are means ± SEM from different experiments ($n$ = 6); *Indicates different from control ($P < 0.05$). (B) Time-course analysis for effect of HP $10^{-6}$ M treatment on LLC-PK1 $Na^+/K^+$-ATPase activity. Data are means ± SEM from different experiments ($n$ = 6); *Indicates different from control ($P < 0.05$).

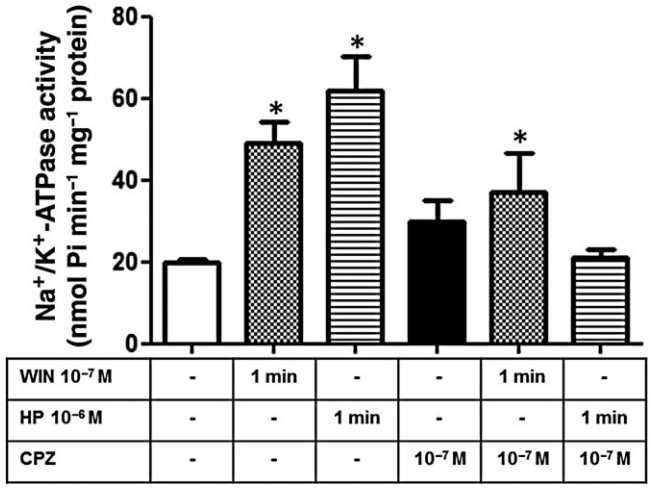
Here we showed that renal epithelial cells express the mRNA for the main ECS enzymes and receptors, which were also detected by immunoblotting (Figures 1 and 2). When LLC-PK1 cells were stimulated by the CB agonist, WIN, there was a clear increase on $Na^+/K^+$-ATPase activity that was completely inhibited by the $CB_1$ antagonist, AM251 (Figure 5). Conversely, the addition of HP, a haemoglobin-derived peptide, and $CB_1$ inverse agonist (Heimann *et al.*, 2007), led to a significant decrease in $Na^+/K^+$-ATPase activity, an effect that was also completely blocked by AM251 (Figure 5). These initial observations allowed us to demonstrate a potential role for the ECS in renal tissue, as it was able to regulate $Na^+/K^+$-ATPase through the activation of the $CB_1$ receptor leading to effects on $Na^+$ homeostasis. In spite of the presence of $CB_2$ receptors, we did not investigate their participation in this phenomenon as the treatment with the specific $CB_1$ antagonist (AM251) was enough to completely abolish the effects of WIN and HP on $Na^+/K^+$-ATPase activity (Figures 5 and 8). We do not rule out the involvement of $CB_2$ receptors in other renal functions, and are currently studying its participation in lesion/repair processes.

Various studies have concluded that GPCR-dependent events are important controllers of different renal outcomes and mainly act on proximal tubule cells. These signalling cascades can start either from the apical and/or basolateral cell membranes, leading to a variety of biological effects including the modulation of $Na^+/K^+$-ATPase (Gomes and Soares-Da-Silva, 2002; Efendiev *et al.*, 2003; Peruchetti *et al.*, 2011). Among the different signalling systems already described for kidney tubular cells, we have now shown that the cAMP/PKA, PKC and ERK pathways are involved in the regulation of $Na^+/K^+$-ATPase activity by the ECS. While the incubation of LLC-PK1 cells with HP (30 min) increased the cAMP levels thus suggesting the involvement of a $G_{\alpha s}$, the same was not true when the cells were stimulated by WIN (Figure 8B). These combined observations allowed us to affirm that lipidic and peptide endogenous cannabinoids



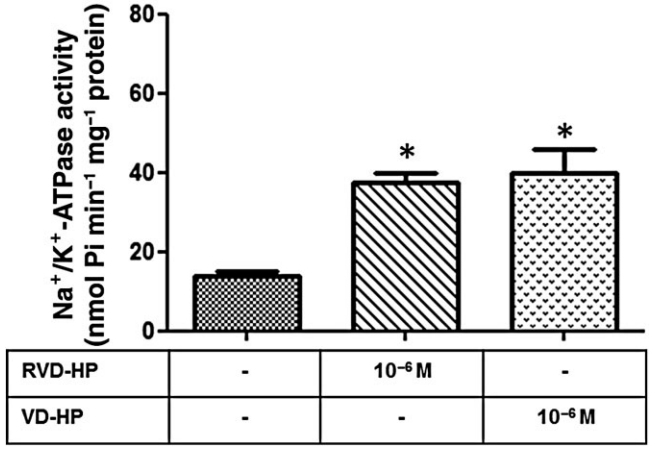

## Figure 5

$CB_1$ receptor is involved in the WIN and HP effects on $Na^+/K^+$-ATPase activity from LLC-PK1 cells. (A) Cells were pretreated for 15 min with the $CB_1$ antagonist, AM251 ($10^{-7}$ M), and then stimulated with $10^{-7}$ M WIN for 1 or 30 min. Data are means ± SEM from different experiments ($n = 6$); *Indicates different from control ($P < 0.05$). (B) Cells were pretreated with AM251 ($10^{-7}$ M) for 15 min before the incubation with HP at $10^{-6}$ M. Data are means ± SEM from different experiments ($n = 6$); *Indicates different from control ($P < 0.05$).




## Figure 6

The TRPV1 channel participates in the modulation of $Na^+/K^+$-ATPase activity induced by WIN and HP in LLC-PK1 cells. Cells were pretreated for 15 min with CPZ ($10^{-7}$ M), a TRPV antagonist, before the incubation with WIN ($10^{-7}$ M) and HP ($10^{-6}$ M). $Na^+/K^+$-ATPase activity was determined 1 min after agonist stimulation. Data are means ± SEM ($n = 6$); *Indicates different from control ($P < 0.05$).

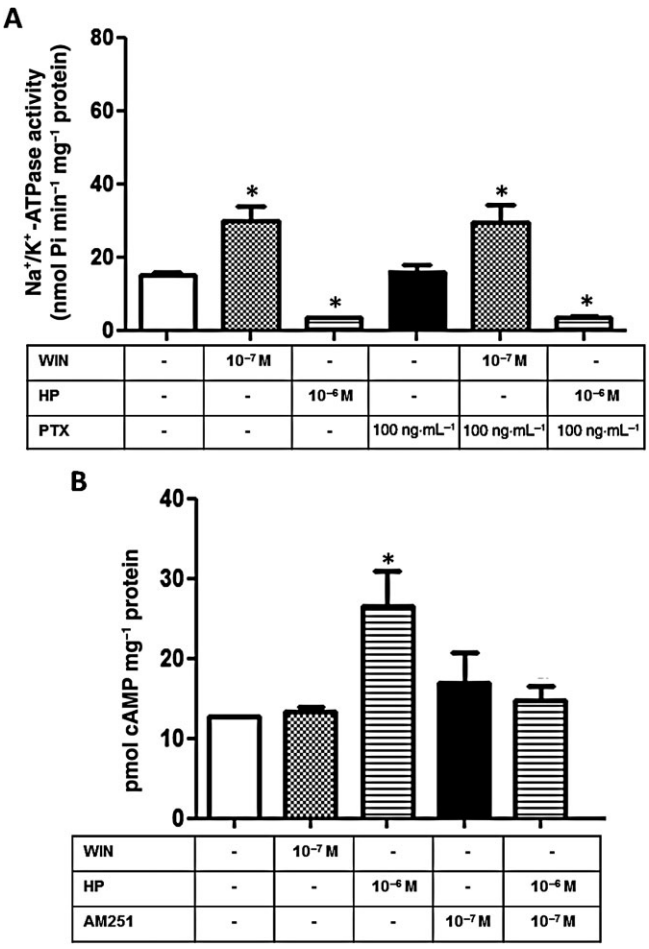



## Figure 7

Effect of endogenous peptides on $Na^+/K^+$-ATPase activity in LLC-PK1 cells. Cells were incubated with RVD-HP and VD-HP ($10^{-6}$ M), and $Na^+/K^+$-ATPase activity was determined after 30 min. Data are means ± SEM ($n = 6$); *Indicates different from control ($P < 0.05$).

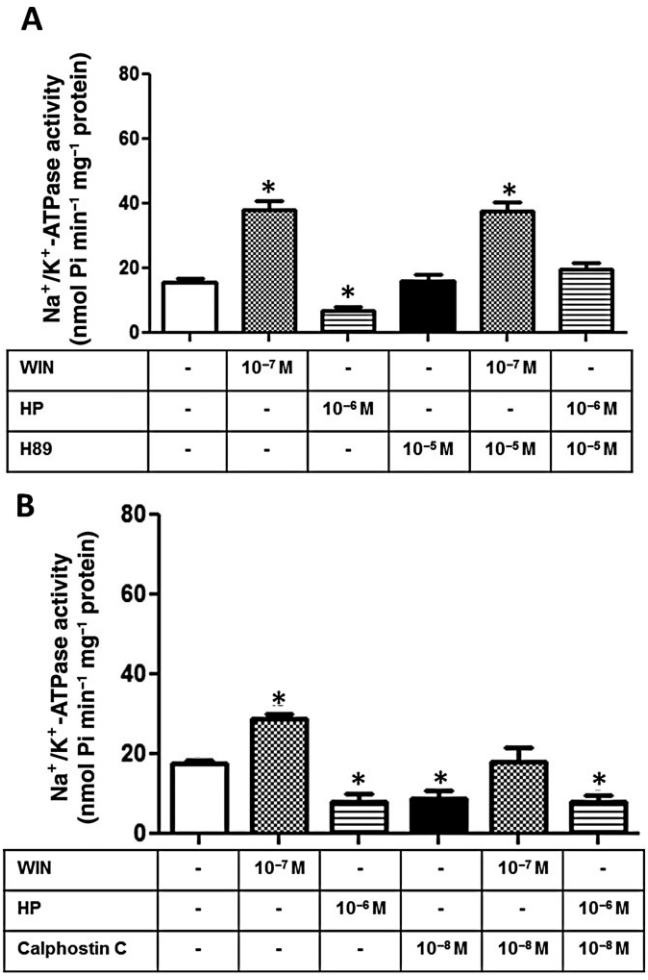
could trigger different pools of $CB_1$ receptors, thus stimulating different signalling pathways resulting in different effects on the $Na^+/K^+$-ATPase. Little is known about how HP interacts with $CB_1$ receptors (Scrima *et al.*, 2010) and how lipidic and peptides from the ECS differentially activate CB receptors. One explanation could be that endogenous lipids (AEA, 2-AG, etc) and RVD-HP or VD-HP (the endogenous peptides that function as selective agonists for $CB_1$ receptors *in vivo*; Gomes *et al.*, 2010), interact with different binding sites on the $CB_1$ receptor. Our results support this view, as we observed that H89 abolished the inhibitory effect of HP on $Na^+/K^+$-ATPase activity, but had no effect on WIN treated cells. It was also demonstrated that while HP decreased the phosphorylation levels of ERK on LLC-PK1 cells, the same was not observed for WIN. Conversely, we observed that the PKC pathway also plays a role in the modulation of $Na^+/K^+$-ATPase activity induced by the ECS, as $10^{-8}$ M calphostin C blocked the stimulation of $Na^+/K^+$-ATPase induced by WIN, but had no effect on the inhibitory effect of HP (Figure 9B).

It is worth mentioning that activation of $CB_1$ receptors has been shown to be involved in inflammation and cell death in different experimental models of disease, including kidney diseases. Feizi *et al.* (2008) showed that endocannabinoid analogues could prevent the appearance of characteristic lesions present in kidneys due to ischaemia and reperfusion injury. In addition, Mukhopadhyay *et al.* (2010) elegantly demonstrated that genetic deletion or pharmacological inhibition of $CB_1$ receptors, either by AM281 or




## Figure 8

HP effect on $Na^+/K^+$-ATPase activity in LLC-PK1 cells involves an increase in cAMP levels. (A) Cells were pretreated for 24 h with PTX (100 ng·mL$^{-1}$) before the incubation with WIN ($10^{-7}$ M) or HP ($10^{-6}$ M). $Na^+/K^+$-ATPase activity was determined after 30 min incubation. Data are means ± SEM from different experiments ($n$ = 6); *Indicates different from control ($P$ < 0.05). (B) cAMP quantification was performed as described under Methods after 30 min incubation with WIN ($10^{-7}$) or HP ($10^{-6}$ M). Where indicated, cells were pretreated with the $CB_1$ antagonist AM251 ($10^{-7}$ M) for 15 min before the addition of WIN or HP. Data are means ± SEM from different experiments ($n$ = 6); *Indicates different from control ($P$ < 0.05).

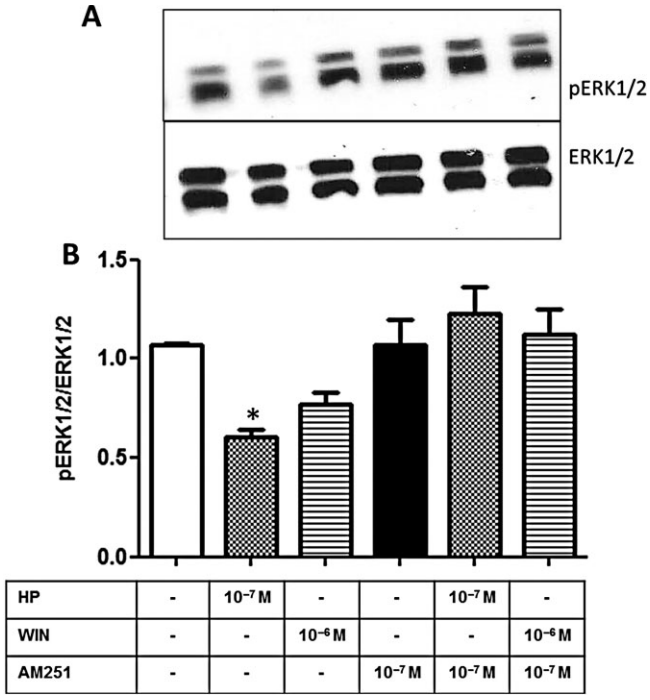



## Figure 9

Involvement of PKA and PKC in the modulation of $Na^+/K^+$ -ATPase activity induced byHP and WIN. (A) When indicated, cells were pretreated for 15 min with H89 ($10^{-5}$ M), a PKA cell-permeable inhibitor, before the incubation with WIN ($10^{-7}$ M) or HP ($10^{-6}$ M). $Na^+/K^+$-ATPase activity was determined after 30 min incubation. Data are means ± SEM from different experiments ($n$ = 6); *Indicates different from control ($P$ < 0.05). (B) When indicated, cells were pretreated for 15 min with, calphostin C ($10^{-8}$ M), a PKC inhibitor, before the incubation with WIN ($10^{-7}$ M) or HP ($10^{-6}$ M). $Na^+/K^+$-ATPase activity was determined after 30 min incubation. Data are means ± SEM from different experiments ($n$ = 6); *Indicates different from control ($P$ < 0.05).

SR141716, significantly prevented some of the harmful effects induced by the chemotherapeutic drug cisplatin on renal remodelling, dysfunction, oxidative/nitrosative stress and cell death pathways, which are very common in this well studied model of kidney injury. Importantly, Barutta and co-workers (2010) showed that $CB_1$ blockers are able to protect the kidney in diabetic rats. In this regard, our data indicate that the ECS is an important target for the prevention of kidney injuries and provide further evidence that HP, an inverse agonist of the $CB_1$ receptor, might play an important role in kidney diseases, probably activating cAMP/PKA pathways, as shown for the LLC-PK1 cells.

Cannabinoids interact with other proteins (Jenkin *et al.*, 2010), such as TRPV1, a member of the TRPV group of cation channels, stimulated by noxious and inflammatory signals such as capsaicin (Di Marzo and De Petrocellis, 2012). Therefore, it is not difficult to imagine that the rapid effects of cannabinoids on the $Na^+/K^+$-ATPase from LLC-PK1 cells could be due to their interaction with a non-selective cation channel. Indeed, these cells express TRPV1 on their membranes (Figures 1 and 2), and CPZ treatment completely abolished the effects of WIN or HP when incubated for 1 min (Figure 6). However, intramedullary infusion of methanandamide in the kidney has been shown to increase the urinary volume and consequently reduce blood pressure by activating $CB_1$ receptors in a TRPV1-independent manner (Li and




## Figure 10

HP decreases ERK1/2 phosphorylation in LLC-PK1 cells. Whole cell homogenates were obtained from cultures treated with WIN ($10^{-7}$ M), HP ($10^{-6}$ M), in the absence or presence of the $CB_1$ antagonist AM251 ($10^{-7}$ M) for 30 min, as depicted. SDS-PAGE and immunoblotting were performed according to Methods. (A) Representative image of pERK1/2 and ERK 1/2 immunodetection. (B) Graph shows the ratio between pERK/ERK after densitometric analysis of the chosen bands in the different experimental situations. Data are means ± SEM from different experiments ($n$ = 6); *Indicates different from control ($P$ < 0.05).

Wang, 2006). Also, Jenkin *et al*. (2010) demonstrated that the mRNA and protein for $CB_1$, $CB_2$ and TRPV1 receptors are expressed in human proximal tubular cells (HK2), and that $CB_1$ receptor activation induced hypertrophy and apoptosis. In contrast, the treatment of HK2 cells with $CB_2$ (AM630) and TRPV1 (SB366791) antagonists increased hypertrophy (Jenkin *et al.*, 2010). Our data on pig proximal tubular cells corroborate these results obtained with human proximal tubule HK2 cells, where different receptors regulate cellular functions by activating distinct signalling pathways.

A notable regulatory role for PKA and PKC on $Na^+$ active transporters has already been demonstrated in kidney tubular cells. PKC promotes the translocation of the $Na^+/K^+$-ATPase into the basolateral membrane and modulates the $Na^+$ influx (Budu *et al.*, 2002), whereas PKA phosphorylates the $Na^+/K^+$-ATPase leading to a decrease in its activity (Cheng *et al.*, 1999). In the present study, we showed that both the PKA and PKC cascades are also triggered by the cannabinoid receptor ligands in a different manner: whereas WIN seems to increase $Na^+/K^+$-ATPase activity through the PKC pathway, whereas the inhibitory effect of HP seems to be mediated by PKA (Figure 9). It was previously shown that the activation of ERK 1/2 can be correlated to an increase in kidney $Na^+/K^+$-ATPase activity (Gildea *et al.*, 2012). Here, we showed that HP promoted a significant decrease in ERK phosphorylation, which was completely abolished by pretreatment of the cells with the $CB_1$ antagonist (Figure 10). As ERKs are known to modulate $Na^+/K^+$-ATPase, we did not investigate the involvement of other MAPKs that can be triggered by cannabinoid signalling. However, we do not rule out their involvement in other kidney processes.

In conclusion our data not only confirm the presence of the ECS in renal proximal tubule cells, but also suggest, for the first time, that this system is involved in the regulation of $Na^+$ re-absorption by direct effects on $Na^+/K^+$-ATPase mediated through different cell signalling cascades. We also hypothesize that the renal ECS interacts with other hormonal systems that control $Na^+$ homeostasis, including the renin-angiotensin and intra-renal dopamine axis. Our results support the view that the ECS in peripheral tissues can act in a 'functionally different' manner from that observed in the CNS. We also showed for the first time that the peptidic cannabinoids have an important role in renal tissue. Compared with other cannabinoid agonists, such as WIN, RVD-HP and VD-HP, HP has the opposite effect in renal tissue; WIN induces its effect by signalling through PKC, whereas HP triggers the cAMP/PKA and ERK pathways. Finally, we showed that the ECS can also regulate the $Na^+/K^+$-ATPase activity by stimulating TRPV1, a mechanism shared by both WIN and HP for short-term effects. Hence, HP appeared to exhibit the same behaviour as WIN after shorter incubation times, which further illustrates the complexity of the effects of the ECS in renal proximal tubule cells.

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## Author contributions

L. S. S. performed experiments, experimental design, discussion of data and written of the manuscript. R. T. D. S. performed experimental design and discussion of data. D. L. performed experiments. C. L. C. S. performed experiments. F. A. I. performed experiments and discussion of data. E. M. performed experiments. V. D. M. performed experimental design, discussion of data and written of the manuscript. A. V. performed discussion of data and written of the manuscript. R. A. M. R. performed experimental design, discussion of data and written of the manuscript. M. E.-L. performed experimental design, discussion of data and written of the manuscript.

## Conflict of interest

Authors declare no conflict of interests.

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