Archives of Biochemistry and Biophysics 535 (2013) 136–142

Contents lists available at SciVerse ScienceDirect

Archives of Biochemistry and Biophysics

journal homepage: www.elsevier.com/locate/yabbi

Protein kinase C-mediated ATP stimulation of Na^+-ATPase activity in LLC-PK1 cells involves a $P2Y_2$ and/or $P2Y_4$ receptor

M. Wengert [a,c,1], M.C. Ribeiro [b,d,1], T.P. Abreu [b,c], R. Coutinho-Silva [b,d], L.R. Leão-Ferreira [c,e], A.A.S. Pinheiro [b,d], C. Caruso-Neves [b,c,*]

[a] Instituto Federal de Educação, Ciência e Tecnologia do Rio de Janeiro, Campus Realengo, 21715-000 Rio de Janeiro, RJ, Brazil
[b] Instituto de Biofísica Carlos Chagas Filho, Universidade Federal do Rio de Janeiro, CCS Bloco G, 21949 Rio de Janeiro, RJ, Brazil
[c] Instituto Nacional de Ciência e Tecnologia em Biologia Estrutural e Bioimagem, Universidade Federal do Rio de Janeiro, 21941-902 Rio de Janeiro, Brazil
[d] Instituto Nacional de Ciência e Tecnologia para Pesquisa Translacional em Saúde e Ambiente na Região Amazônica, Universidade Federal do Rio de Janeiro, 21941-902 Rio de Janeiro, Brazil
[e] Instituto de Biologia, Universidade Federal Fluminense, Campus do Valonguinho, 24020-150 Niterói, RJ, Brazil

ARTICLE INFO

Article history:
Received 30 January 2013
and in revised form 14 March 2013
Available online 6 April 2013

Keywords:
Purinergic receptor
Kidney
Renal function
Renal sodium excretion

ABSTRACT

ATP-activated P2Y receptors play an important role in renal sodium excretion. The aim of this study was to evaluate the modulation of ATPase-driven sodium reabsorption in the proximal tubule by ATP or adenosine (Ado). LLC-PK1 cells, a model of porcine proximal tubule cells, were used. ATP (10^{-6} M) or Ado (10^{-6} M) specifically stimulated Na^+-ATPase activity without any changes in ($Na^+ + K^+$)-ATPase activity. Our results show that the Ado effect is mediated by its conversion to ATP. Furthermore, it was observed that the effect of ATP was mimicked by UTP, ATPγS and 2-thio-UTP, an agonist of $P2Y_2$ and $P2Y_4$ receptors. In addition, ATP-stimulated Na^+-ATPase activity involves protein kinase C (PKC). Our results indicate that ATP-induced stimulation of proximal tubule Na^+-ATPase activity is mediated by a PKC-dependent $P2Y_2$ and/or $P2Y_4$ pathway. These findings provide new perspectives on the role of the effect of P2Y-mediated extracellular ATP on renal sodium handling.

Introduction

Renal tubular cells secrete [2]ATP, which promotes autocrine and paracrine activity, modulating renal sodium excretion through specific P2 receptors: ionotropic P2X and metabotropic P2Y [1–4]. On the other hand, extracellular ATP can be hydrolyzed by sequential reactions involving ecto-ATPases and 5′-nucleotidase forming adenosine (Ado) [5]. Similar to ATP, Ado is also able to modulate renal function through specific P1 receptors. P1 receptors are members of the G protein-coupled receptor superfamily (GPCR) and it is further subclassified into A_1, A_{2A}, A_{2B}, and A_3 receptors [6]. Therefore, the final effect of ATP depends on coordinated mechanisms that involve different receptors and ecto enzymes.

The crucial roles of Ado and ATP in renal sodium excretion during physiologic and pathophysiologic events have been reported previously [7–9]. However, depending on the receptor type involved, ATP or Ado could have contradictory effects on renal sodium excretion [6,9]. Infusion of $P2Y_2$ and $P2Y_4$ receptor agonists in $P2Y_2^{-/-}$ knockout mice indicate that these receptors have opposite effects on blood pressure and renal sodium excretion [10]. In addition, it has been shown that $P2Y_2$ decreases NKCC2 transport in the thick ascending limb and conductance in the renal epithelial sodium channel (ENaC) [11,12]. In addition, it has been proposed that P2X receptors have a role in renal sodium excretion [13]. These receptors were observed to modulate ENaC in distal segments [14]. Similarly, several studies have shown that Ado receptors have an important role in renal sodium excretion through modulation of specific sodium transporters located along the nephron [6].

It is well known that proximal tubule cells are responsible for reabsorption of 70% of the total sodium filtered in the glomerulus.

* Corresponding author at: Instituto de Biofísica Carlos Chagas Filho, Universidade Federal do Rio de Janeiro, CCS Bloco G, 21949 Rio de Janeiro, RJ, Brazil. Fax: +55 21 2280 8193.
E-mail address: caruso@biof.ufrj.br (C. Caruso-Neves).

[1] These authors contributed equally to the study.

[2] *Abbreviations used:* Ado, adenosine; AMP, adenosine 5′-monophosphate disodium salt; ATP, adenosine 5′-triphosphate disodium salt hydrate; DMEM, Dulbecco's modified Eagle's medium; DMPX, 3,7-dimethyl-1-propargylxanthine; DPCPX, 8-cyclopentyl-1,3-dipropylxanthine; ENaC, epithelial sodium channel; GPCR, G protein-coupled receptor; HEPES, 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; MRS1523, 2,3-diethyl-4,5-dipropyl-6-phenylpyridine-3-thiocarboxylate-5-carboxylate; PBS, phosphate-buffered saline; PKA, protein kinase A; PKC, protein kinase C; PMA, phorbol myristate acetate; PPADS, pyridoxal phosphate-6-azo(benzene-2,4-disulfonic acid); UTP, uridine 5′-triphosphate trisodium salt dihydrate; UDP, uridine 5′-diphosphate disodium salt hydrate; ATPγS, adenosine 5′-[γ-thio]triphosphate tetralithium salt; 2-thio-UTP tetrasodium salt, 2-thiouridine 5′-triphosphate tetrasodium salt; MRS 2693 trisodium salt, 5-iodouridine-5′-*O*-diphosphate trisodium salt; TCA, trichloroacetic acid; TLC, thin-layer chromatography

0003-9861
http://dx.doi.org/10.1016/j.abb.2013.03.013

Thus, any change in sodium reabsorption in proximal tubule cells could lead to variations in renal sodium excretion even with compensatory reabsorption of sodium in distal segments of the nephron [15]. This idea is supported by the observation that there is strict correlation between renal sodium excretion and proximal tubule sodium reabsorption in some pathologic conditions such as primary hypertension [16]. Furthermore, it has been shown that proximal tubule cells express P1, P2Y, and P2X receptors [1], and these receptors modulate sodium transporters in the proximal tubule such as NHE [17] and bicarbonate cotransporters [18]. However, the activity of these cotransporters depends on the gradient previously created by sodium pumps.

Transcellular proximal tubule sodium reabsorption is mainly driven by 2 sodium pumps: ouabain-sensitive ($Na^+ + K^+$)-ATPase activity and furosemide-sensitive Na^+-ATPase, called the second sodium pump [19,20]. In a previous study, it was shown that 10^{-3} M ATP inhibits ($Na^+ + K^+$)-ATPase [21]. Na^+-ATPase was recently cloned in enterocytes and its role in renal sodium excretion has been proposed [22]. Our group showed that this enzyme is a target for receptor-mediated nucleoside activity in the basolateral membrane of proximal tubule cells [7,23]. However, modulation of proximal tubule Na^+-ATPase activity by P2 receptor-mediated ATP has not yet been studied.

The aim of the present work was to investigate the modulation of proximal tubule ($Na^+ + K^+$)-ATPase and Na^+-ATPase activities by luminal ATP in cultured LLC-PK1 cells, a well-known model of porcine proximal tubule cells. It was observed that P2Y-mediated ATP stimulated Na^+-ATPase activity through the activation of protein kinase C (PKC). These results provide new perspectives on our understanding of the role of ATP on renal sodium excretion.

Materials and methods

Materials

ATP, ouabain, furosemide, HEPES, Tris, Ado, hypoxanthine, adenine, 5′-iodotubercidin, pyridoxal phosphate-6-azo(benzene-2,4-disulfonic acid) (PPADS), UTP, UDP, ATPγS, AMP, and ADP were purchased from Sigma Chemical Co. (St. Louis, MO). 8-Cyclopentyl-1,3-dipropylxanthine (DPCPX), 3,7-dimethyl-1-propargylxanthine (DMPX), MRS1523 were purchased from Research Biochemicals International (Natick, MA). 2-Thio-UTP and MRS2693 were purchased from Tocris Bioscience. Rabbit polyclonal antibodies against $P2Y_2$ and $P2Y_4$ receptors were purchased from Santa Cruz Biotechnology Inc., Santa Cruz, CA (Cat. # sc-20124), and Alomone Labs, Jerusalem, Israel (Cat. #APR-006), respectively. Polyclonal β-actin antibodies were purchased from Cell Signaling Technology, Danvers, MA (Cat. #4967). [^{3}H]Ado labeled in the 2-position of the adenine moiety (22 Ci/mmol) was purchased from Amersham Biosciences (UK). Calphostin C was purchased from Calbiochem (USA). [^{32}P]Pi was obtained from the Institute of Energetic and Nuclear Research (São Paulo, SP, Brazil). All other reagents were of the highest purity available. All solutions were prepared with deionized water. [γ-^{32}P]ATP was prepared as previously described [13].

Cell culture

The porcine proximal tubule cell line LLC-PK1 (American Type Culture Collection, Rockville, MD) was maintained in low-glucose Dulbecco's modified Eagle's medium (DMEM) complemented with 10% fetal bovine serum and 1% penicillin/streptomycin (37 °C and 5% CO_2). One-day post-confluence cells were used in all experiments, usually 3 days after seeding. Cells were kept overnight in serum-free medium before the experiments.

Analysis of the metabolism of Ado in LLC-PK1 cells by thin-layer chromatography

Analysis of the metabolism of [^{3}H]Ado in LLC-PK1 cells was carried out as previously reported by Assaife-Lopes et al. [23] with some modifications. Briefly, confluent cultures of LLC-PK1 cells grown in 24-well plates were incubated with serum-free culture medium containing 10^{-7} M [^{3}H]Ado at 37 °C for 30 min. Each culture supernatant was then collected for analysis by thin-layer chromatography (TLC) and 20% trichloroacetic acid (TCA) was added to the cell monolayers. The cells were harvested from the plates and centrifuged for 15 min at 15,000 rpm in an Eppendorf centrifuge at 4 °C. Aliquots (60 μl) of the supernatant from each cell homogenate as well as culture supernatants were spotted on the TLC plate (silica gel 60 F254, 16 × 4 cm, 0.25-mm layer thickness; Merck). The mobile phase consisted of methanol, ethyl acetate, butanol, ammonium hydroxide (3:4:7:4, by vol.). An aliquot (30 μl) of a mix containing 5 mM of each purine standard (Ado, inosine, hypoxanthine, adenine nucleotides, and adenine) was also spotted over each sample. After running for 15 cm, the organic phase of the TLC was evaporated to dryness at room temperature. The areas corresponding to metabolites derived from [^{3}H]Ado were visualized by reflection of ultraviolet light by co-migration with the respective purine standards, scraped from the plates, and transferred to a scintillation vial containing 0.4% diphenyloxazole and toluene, Triton X-100, water (8:4:1, by vol.). The radioactivity contained in each area was quantified by liquid scintillation counting (Packard Tri-Carb 2100 TR, Downers Grove, IL).

Cell homogenate

Confluent cultures of LLC-PK1 cells grown in 6-well plates were washed with phosphate-buffered saline (PBS) and pre-incubated with 2 ml of serum-free culture medium (starved) overnight. The cells were incubated with different drugs as indicated in the figures. After incubation, the cell monolayers were washed with PBS and harvested from the plates with 2 ml of PBS. After centrifugation (1000 rpm), cell pellets were resuspended in homogenizing buffer (0.1% deoxycholic acid, 1 mM EDTA, 20 mM HEPES–Tris (pH 7.0), 250 mM sucrose) and incubated for 30 min at 4 °C. The protein concentration of the cell homogenates was determined by the Folin phenol method [24] using bovine serum albumin as a standard.

ATPase activity assays

ATPase activity was measured according to the method described by Grubmeyer and Penefsky [25]. The composition of the assay medium (0.1 ml) was 10 mM $MgCl_2$, 30 mM KCl, 20 mM HEPES–Tris (pH 7.0), 120 mM NaCl, and 5 mM ATP-Na^+ (specific activity of approximately 10^4 Bq/nmol ATP). The ATPase activity of the cell homogenates (the protein concentration in the assay was 0.2–0.3 mg/ml) was determined by incubating for 20 min at 37 °C. The reaction was stopped by the addition of HCl-activated charcoal (0.1 N). An aliquot of the supernatant was obtained after centrifugation of the HCl-activated charcoal suspension for 5 min at 2000 rpm in a clinical centrifuge. [^{32}P]Pi was measured by liquid scintillation counting (Packard Tri-Carb model A2100TR, Downers Grove, IL). The ($Na^+ + K^+$)-ATPase activity was determined from the difference between [^{32}P]Pi released in the absence and in the presence of 1 mM ouabain. The Na^+-ATPase activity was determined from the difference between [^{32}P]Pi released in the absence and in the presence of 2 mM furosemide (an Na^+-ATPase specific inhibitor), both in the presence of 1 mM ouabain (an ($Na^+ + K^+$)-ATPase specific inhibitor) [19,20].

Protein kinase A and C activities

Protein kinase A (PKA) and PKC activities in the cell homogenates were determined as the difference in the transfer of ^{32}Pi from [γ-^{32}P]ATP into histone in the absence and in the presence of PKA or PKC selective inhibitors, as previously described [7,23]. The composition of the reaction medium (0.1 ml) was 4 mM $MgCl_2$, 20 mM HEPES–Tris (pH 7.0), 1.5 mg/ml histone, and 0.7 mg/ml protein. The reaction was started by the addition of [γ-^{32}P]ATP (magnesium salt, 7 μCi/μmol), at a final concentration of 10 μM. After 10 min, the reaction was stopped by the addition of 20% TCA and the samples were immediately placed on ice. The samples were filtered using Millipore filters (0.45 μm) and washed with ice-cold solution containing 20% TCA and 2 mM phosphate buffer (pH 7.0). The radioactivity was measured by liquid scintillation counting (Packard Tri-Carb 2100 TR). PKA activity was considered to be the difference between the level of ^{32}Pi-phosphorylated protein in the absence and in the presence of 10^{-8} M PKAi (PKA inhibitor) [32]. PKC activity was considered to be the difference between the level of ^{32}Pi-phosphorylated protein in the absence and in the presence of 10^{-8} M calphostin C (a PKC inhibitor) [26,27].

Immunoblotting

Immunoblotting was carried out to investigate the expression of $P2Y_2$ and $P2Y_4$ receptors in LLC-PK1 cells. Fifty micrograms of cell homogenate were loaded onto 9% SDS–PAGE gels and transferred to polyvinyl difluoride membranes [28]. The membranes were blocked with TBS 0.05% Tween 20 (TBST) plus 5% non-fat dry milk for 1 h at room temperature. After 3 washes with ice-cold 0.05% TBST, membranes were incubated for 1 h with rabbit polyclonal antibodies raised against human $P2Y_2$ or rat $P2Y_4$ receptors, diluted 1:300 in 0.05% TBST plus 5% non-fat dry milk. This procedure was followed by 3 consecutive washes with ice-cold 0.05% TBST.

Thereafter, membranes were incubated with anti-rabbit IgG horseradish peroxidase-conjugated antibody diluted 1:40,000 in 0.05% TBST plus 5% non-fat dry milk and then washed 3 more times with 0.05% TBST. The blots were developed using ECL-plus reagent according to the manufacturer's instructions.

Statistical analysis of the data

The data were analyzed by 2-way analysis of variance, considering the treatments as factors. The significance of the differences was verified by the Bonferroni *t*-test.

Results

ATP stimulates Na^+-ATPase activity in LLC-PK1 cells

It is well known that proximal tubule cells secrete ATP to the luminal side [3,4]. The effect of ATP could be correlated to its direct interaction with specific P2 receptors or mediated by metabolites such as Ado through specific P1 receptors. In the first experimental group, we studied the possible role of extracellular ATP or Ado on Na^+-dependent ATPase activities (Na^+-ATPase and ($Na^+ + K^+$)-ATPase). LLC-PK1 cells were pre-incubated with serum-free culture medium (starved) overnight. Cells were washed out and 10^{-6} M ATP or 10^{-6} M Ado was added to the medium for a further 30 min at 37 °C. Incubation with ATP or Ado increased the activity of Na^+-ATPase but did not change the activity of ($Na^+ + K^+$)-ATPase (Fig. 1A).

In the next step, we decided to investigate if Ado modulates Na^+-ATPase activity through interaction with specific P1 receptors. LLC-PK1 cells were incubated with Ado in the presence of classic P1 receptor-selective antagonists. Our results show that the stimulatory effect on Na^+-ATPase induced by 10^{-6} M Ado was not affected by 10^{-7} M DPCPX, 10^{-6} M DMPX, or 10^{-9} M MRS1523 (A_1, A_2 or A_3 receptor-selective antagonists, respectively) (Fig. 1B). The isolated P1 receptor-selective antagonists tested here did not affect the activity of Na^+-ATPase (data not shown). These data show that the effect on Na^+-ATPase induced by Ado is not mediated by P1 receptors.

Based on the results, we can hypothesize that Ado is being converted to ATP rather than the effect of ATP being mediated by its conversion to Ado. To test this hypothesis, LLC-PK1 cells were incubated with [^{3}H]Ado and the levels of ^{3}H-labeled adenine nucleotides (AMP, ADP, ATP) were determined by TLC. The level of ^{3}H-labeled adenine nucleotides was significantly increased in the extracellular medium (Fig. 2A). In addition, ^{3}H-labeled hypoxanthine, inosine, and adenine were also increased after the addition of [^{3}H]Ado to the culture medium. We tested the effect of hypoxanthine and adenine on the activity of Na^+-ATPase (Fig. 2B). Neither of these compounds was able to change the enzyme activity, indicating that they do not mediate the stimulatory effect of Ado.

It is possible that sequential phosphorylation of Ado leading to the formation of ATP is involved in the stimulatory effect of Ado. Phosphorylation of Ado to AMP is a prerequisite for the Ado-induced effect as shown by incubation of LLC-PK1 cells with Ado in the presence of 5′-iodotubercidin, a specific inhibitor of adenosine kinase. 5′-iodotubercidin (10^{-4} M) completely blocked the stimula-

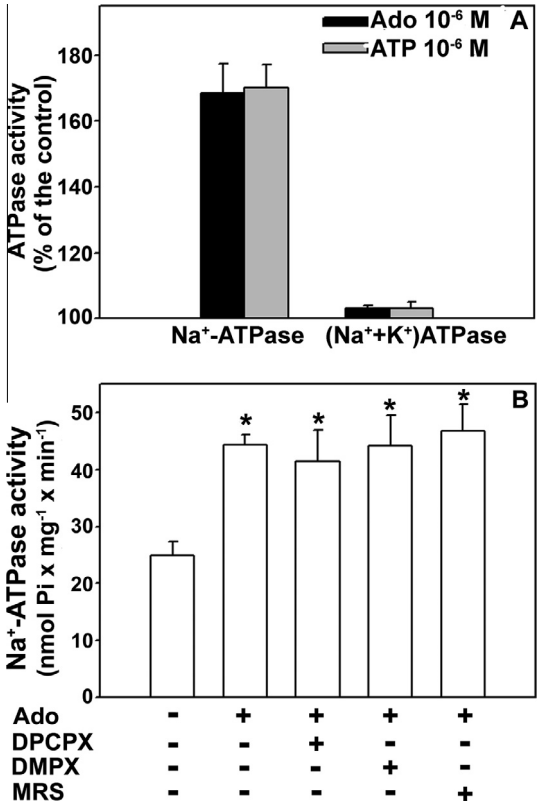

Fig. 1. ATP-induced effects on sodium-dependent ATPase activity in LLC-PK1 cells. (A) ATP (10^{-6} M) and Ado (10^{-6} M) stimulated Na^+-ATPase activity but did not affect ($Na^+ + K^+$)-ATPase activity. Black bars represent the mean values for each enzyme activity in the presence of Ado; white bars represent the mean values for each enzyme activity in the presence of ATP. (B) Ado-induced stimulation of Na^+-ATPase activity was not mediated by P1 receptor subtypes: DPCX, DMPX, and MRS1523 are A_1, A_2, and A_3 receptor-selective antagonists, respectively. ATPase activity was measured as described in the Materials and methods section. The results are expressed as mean ± SE. *Statistically significant compared with control ($P < 0.05$, $n = 8$).

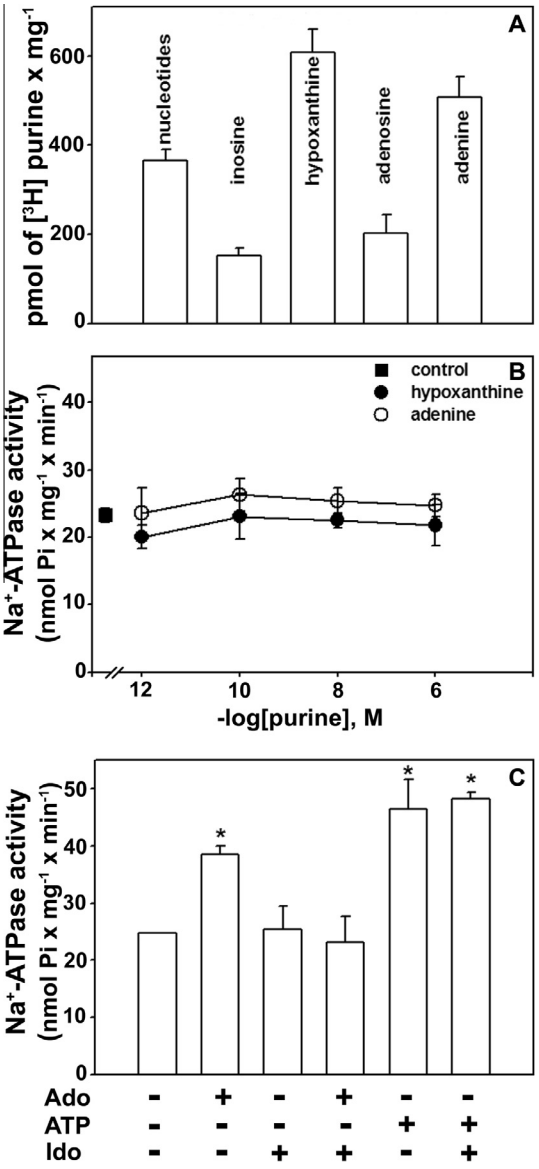

Fig. 2. Ado-induced stimulation of Na^+-ATPase activity involves nucleoside phosphorylation to ATP. (A) LLC-PK1 cells were incubated with serum-free culture medium containing 10^{-7} M [^{3}H]Ado at 37 °C for 30 min. After incubation, the extracellular culture medium was fractionated by TLC and ^{3}H-labeled purine derivatives ([^{3}H]adenine, [^{3}H]inosine, [^{3}H]hypoxanthine, and [^{3}H]adenine nucleotides) were quantified by liquid scintillation, as described in the Materials and methods section. (B) Hypoxanthine and adenine did not affect Na^+-ATPase activity. (C) Adenosine-induced stimulation of Na^+-ATPase activity involved adenosine kinase activity: iodotubercidin (Ido, 10^{-4} M), an adenosine kinase inhibitor, prevented the effect induced by Ado (10^{-6} M) but did not affect the ATP (10^{-6} M)-induced stimulation of Na^+-ATPase activity. The ATPase activity was measured as described in the Materials and methods section. The results are expressed as mean ± SE. *Statistically significant compared with control ($P < 0.05$, $n = 8$).

tory effect on Na^+-ATPase activity induced by Ado but it did not affect the stimulatory effect induced by ATP (Fig. 2C).

The P2 receptor-mediated stimulatory effect of ATP on Na^+-ATPase activity

To investigate the involvement of P2 receptors in the stimulatory effects induced by ATP on Na^+-ATPase activity, LLC-PK1 cells were incubated simultaneously with 10^{-6} M ATP, 10^{-6} M Ado, or 10^{-6} M non-hydrolysable ATP (ATPγS) in the presence or absence of 5×10^{-5} M PPADS, a nonspecific P2 receptor antagonist. Our results show that PPADS blocked the stimulatory effects induced by ATP, Ado, or ATPγS on Na^+-ATPase activity (Fig. 3A), indicating a P2 receptor-mediated response for ATP. In addition, ATPγS mimicked the effect of ATP confirming that hydrolysis of ATP is not necessary for its final effect on the enzyme activity. The possibility that basal ATP could be modulating Na^+-ATPase activity in LLC-PK1 cells can be ruled out because PPADS did not have this effect in the absence of added ATP.

We showed that part of Ado has already been converted to adenine nucleotides (AMP, ADP, ATP) after 30 min of incubation. Furthermore, in the time course experiments we observed that Ado metabolism in luminal side of LLC-PK1 is maximal after 10 min of incubation (data not shown). Then, in another experimental approach cells were incubated with 10^{-6} M Ado for 30 min. The culture medium (conditioned medium) was then collected and added to another culture of LLC-PK1 cells and Na^+-ATPase activity was measured (Fig. 3B). The conditioned medium increased the enzyme activity by 55%, similar to the effect observed in the presence of added ATP. In addition, the stimulatory effect induced by the conditioned medium was abolished by 5×10^{-5} M PPADS.

Our data demonstrate that the stimulatory effect induced by Ado depends on its conversion to ATP which is involved in the modulation of proximal tubule Na^+-ATPase activity.

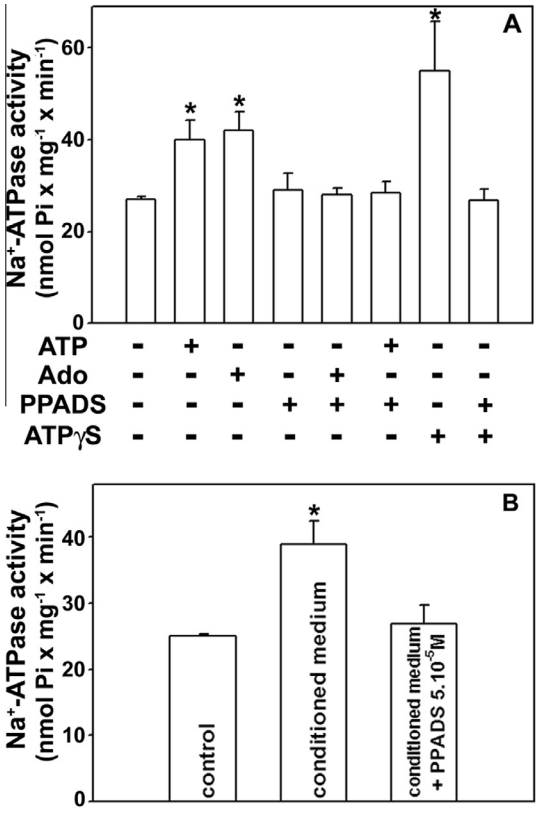

Fig. 3. The P2 receptor antagonist PPADS blocked the stimulatory effects induced by ATP, ATPγS, and Ado on Na^+-ATPase activity. (A) The first bar shows the enzyme activity in the absence of either Ado or non-hydrolysable ATP (ATPγS) or ATP; PPADS (5×10^{-5} M), a P2 receptor-selective antagonist, prevented the stimulatory effects on Na^+-ATPase activity induced by Ado (10^{-6} M), ATPγS (10^{-6} M), and ATP (10^{-6} M). (B) PPADS blocks the stimulatory effect of conditioned medium on Na^+-ATPase activity: confluent LLC-PK1 cells were incubated in 10^{-6} M Ado for 30 min; the culture medium (conditioned medium) was then transferred to a different monolayer of LLC-PK1 cells and incubated for 30 min in the absence or in the presence of 5×10^{-5} M PPADS. The ATPase activity was measured as described in the Materials and methods section. The results are expressed as mean ± SE. *Statistically significant compared with control ($P < 0.05$, $n = 7$).

The stimulatory effect of ATP on Na^+-ATPase activity involves P2Y receptor-mediated PKC activation

The existence of $P2Y_1$, $P2Y_2$, $P2Y_4$, and $P2Y_6$ in proximal tubules has been demonstrated previously [29,30]. Involvement of the P2Y receptor subtype on the stimulatory effect of ATP on Na^+-ATPase activity was demonstrated using UTP, a classic agonist for P2Y receptors (Fig. 4A). UTP (10^{-6} M) stimulated Na^+-ATPase activity in a similar manner to ATP. Interestingly, 10^{-6} M ADP, which is able to bind to the $P2Y_1$ receptor, did not change the Na^+-ATPase activity. The possible role of the $P2Y_6$ receptor was investigated using UDP (Fig. 4A) and MRS2693 (Fig. 4B), a selective agonists of this receptor [31]. Neither compound changed the enzyme activity, suggesting that $P2Y_6$ is not involved in the stimulatory effect of ATP.

We also tested the effect of 2-thio-UTP, an agonist for $P2Y_2$ (at nanomolar concentration) and $P2Y_4$ (at micromolar concentration) receptors [32]. It was observed that this agonist increased Na^+-ATPase activity at both lower (0.05 μM) and higher (0.5 μM) concentrations (Fig. 4C). The maximal stimulatory effect was similar to that found for 10^{-6} M ATP or 10^{-6} M ATPγS. So far, our results show that the metabotropic $P2Y_2$ and/or $P2Y_4$ subfamily is likely the P2 receptor subtype involved in the modulation of Na^+-ATPase activity by ATP. In addition, the expression of $P2Y_2$ and $P2Y_4$ receptors in LLC-PK1 cells was evaluated by immunoblotting using polyclonal-specific antibodies and revealed expression of both receptors (Fig. 5). The smooth second band observed in $P2Y_2$ blotting probably represents the degradation of the protein. The immunoblotting using only secondary antibody was carried out and the second band does not appear (data not shown).

It has been shown that Na^+-ATPase activity can be modulated by PKA and PKC [7,23,26,27]. In addition, modulation of the PKA and PKC signaling pathways by $P2Y_2$ and $P2Y_4$ receptors has been reported previously [2]. For those reasons, confluent LLC-PK1 cells were incubated with 10^{-6} M ATP or 10^{-6} M Ado for 30 min and cell homogenates were used to measure the activity of PKA and PKC. Our results show that ATP or Ado did not modulate PKA activity (Fig. 6A). In sharp contrast, both ATP and Ado stimulated PKC activity, which was completely blocked by 5×10^{-5} M PPADS. This stimulation was equivalent to that promoted by 10^{-12} M phorbol myristate acetate (PMA), a classic activator of PKCs (Fig. 6B). The possible relationship between the stimulation of PKC and stimulated Na^+-ATPase activity was demonstrated by using 10^{-8} M calphostin C, a specific inhibitor of PKC. The stimulatory effects of ATP on Na^+-ATPase activity were completely blocked by calphostin C (Fig. 6C).

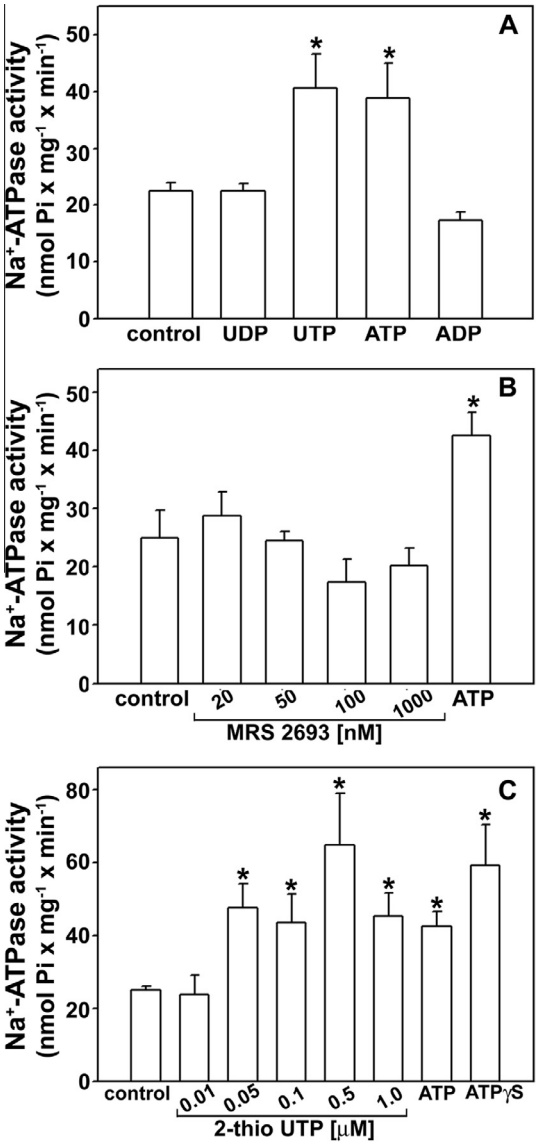

Fig. 4. Involvement of $P2Y_2$/$P2Y_4$ receptor in the stimulatory effect of ATP on Na^+-ATPase activity. (A) ATP (10^{-6} M) and UTP (10^{-6} M), a classic agonist for P2Y receptors, stimulated Na^+-ATPase activity, but UDP (10^{-6} M), a selective agonist of $P2Y_6$ receptor, and ADP (10^{-6} M), which is able to bind to the $P2Y_1$ receptor, did not affect the enzyme activity. (B) The activation of $P2Y_6$ receptor was not able to stimulate Na^+-ATPase activity: LLC-PK1 was incubated with MRS2693 (from 20 to 1000 nM), an agonist of $P2Y_6$, but the Na^+-ATPase activity was not affected. (C) 2-Thio-UTP, an agonist of $P2Y_2$/$P2Y_4$ receptors, stimulated Na^+-ATPase activity: low and high concentrations of 2-thio-UTP stimulated Na^+-ATPase activity in a similar manner. ATPase activity was measured as described in the Materials and methods section. The results are expressed as mean ± SE. *Statistically significant compared with control ($P < 0.05$, $n = 12$).

Discussion

The role of extracellular ATP on cellular functions through specific P2 receptors has been a topic of interest [1,4]. ATP is secreted by renal epithelial cells, through apical and basolateral membranes, but the full effect on the function of these cells is not known. In the present work, we showed that extracellular ATP activates $P2Y_2$ and/or $P2Y_4$ receptors in the plasma membrane of LLC-PK1 cells, stimulating Na^+-ATPase activity through a PKC signalling pathway. These results provide new perspectives on understanding the role of ATP on the sodium transporters located in proximal tubule cells.

We used LLC-PK1 cells, which could be a limitation in determining the final effect of $P2Y_2$ and/or $P2Y_4$ receptor activation on proximal tubule sodium reabsorption and, consequently, renal sodium excretion. However, LLC-PK1 cells have been widely used as a model of proximal tubule cells and the results obtained in these cells are usually reproduced in proximal tubule cells [7,23,33,34].

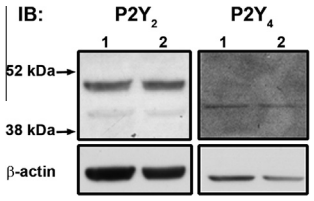

Fig. 5. Immunodetection of $P2Y_2$ and $P2Y_4$ receptors. LLC-PK1 proteins were separated by 9% PAGE–SDS. $P2Y_2$ and $P2Y_4$ receptors were detected by immunoblotting using specific polyclonal antibodies (in duplicate), as described in the Materials and methods section. β-actin was used as a loading control. Lane 1 and 2 represent different preparations of the LLC-PK1 cells.

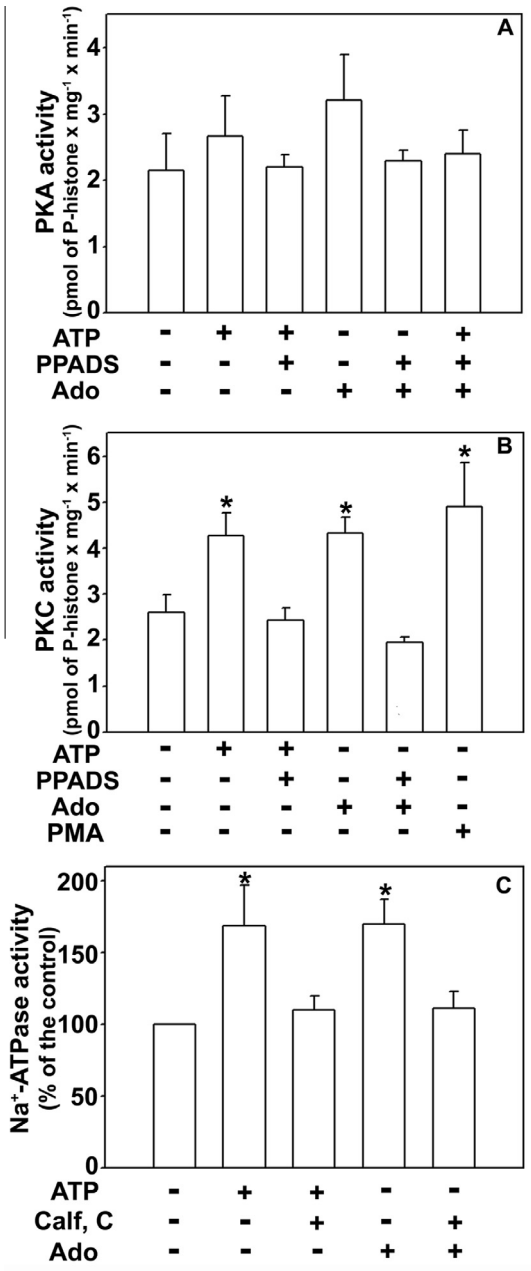

Fig. 6. Stimulatory effects induced by ATP and Ado on Na^+-ATPase activity involved PKC activation. (A) Neither ATP nor Ado modulated PKA activity. (B) ATP and Ado stimulated PKC activity in a PPDAS-sensitive manner and these stimulatory effects were similar to those of PMA, an activator of PKC. (C) Stimulatory effects induced by ATP and Ado on Na^+-ATPase activity were blocked by calphostin C (Calph C), a specific inhibitor of PKC. The ATPase and protein kinase activities were measured as described in the Materials and methods section. The results are expressed as mean ± SE. *Statistically significant compared with control ($P < 0.05$, $n = 6$).

Adenine nucleotides are reversibly converted to Ado by intracellular and extracellular (ecto-) enzymes, such as nucleoside triphosphate diphosphohydrolases (NTPDases), nucleotide pyrophosphatase/phosphodiesterase (NPPases) and 5′-nucleotidase [5,34]. A functional role of the extracellular unidirectional metabolism of adenine nucleotides into Ado in the kidney has been suggested previously [35]. In a previous study, our group showed that Ado added to the basolateral membrane modulates the activity of Na^+-ATPase activity via P1 receptors: A_1 and A_2. Activation of A_1 was reported to lead to a decrease in enzyme activity and interaction with A_2 increases it [7,23]. In addition, this biphasic effect depends on the inhibition or activation of the cAMP/PKA pathway. In contrast, in the present work, ATP was found to be a mediator of the luminal Ado-induced stimulatory effect on Na^+-ATPase activity. These data indicate that there is a differential metabolism of Ado at the luminal and basolateral membranes of proximal tubule cells.

Ouabain-insensitive Na^+-ATPase was recently cloned in enterocytes [22]. However, even before it was cloned, the evidence indicated that this enzyme is involved in sodium transport in different cell types including proximal tubule cells [26,27]. Malnic et al. [36] observed that furosemide inhibits Na^+ reabsorption in the proximal tubule. It has been proposed that Na^+-ATPase is involved in fine tuning Na^+ reabsorption in the proximal tubule [23]; this is supported by the observation that change in Na^+-ATPase leads to significant changes in sodium reabsorption in proximal tubule cells in spontaneously hypertensive rats [37]. In addition, it has been shown that this enzyme is a target to several hormones and autacoids involved in the modulation of renal sodium excretion [7,23,26,27,38,39].

In the present work, we observed that apical ATP 10^{-6} M affected Na^+-ATPase activity specifically but did not change the activity of $(Na^+ + K^+)$-ATPase in LLC-PK1 cells incubated for 30 min. Jin and Hopfer et al. [21] demonstrated that apical ATP 10^{-3} M inhibits $(Na^+ + K^+)$-ATPase activity in WKPT1292 C1.8 cells (Wistar Kyoto rat kidney proximal tubule cells), measured by short-circuit current, in a rapid (1 min) and transient way. These apparently contradictory results can be explained by differences in the ATP concentration, time of incubation, or even due to the methodology used to measure the $(Na^+ + K^+)$-ATPase activity. In addition, these apparent contradictory effects might reveal an important balance between the activity of 2 sodium pumps: at a lower concentration, ATP could stimulate sodium reabsorption through Na^+-ATPase, whereas at a higher level, as a result of cell leakage, ATP could inhibit the massive sodium reabsorption through $(Na^+ + K^+)$-ATPase.

The role of ATP in proximal tubule sodium reabsorption may be related to the concentration of ATP, which binds to different P2X and P2Y receptors expressed in this segment [40]. The following observations indicate that the effect of ATP on the activity of Na^+-ATPase is mediated by $P2Y_2$ and/or $P2Y_4$: (1) UTP, ATPγS, and 2-thio-UTP mimicked the stimulatory effect of ATP on Na^+-ATPase activity; (2) PKC mediates the ATP effect. It is well known that P2Y receptors can trigger a cellular response through activation of PKC [2].

The proximal tubule is the nephron segment with the highest level of secretion of ATP. Veckaria et al. [3] measured the concentration of ATP in the lumen of proximal tubules from rat kidney and found that it ranged from 100 to 300 nmol/l. These values could reach micromolar concentrations depending on the physiologic or pathophysiologic conditions. Because P2Y receptor subtypes have high affinity for ATP, its luminal concentration could be high enough to activate these receptors [2,10]. Our results suggest that ATP secretion at the luminal membrane could be an important mechanism in modulating proximal tubule sodium reabsorption through a $P2Y_2$ and/or $P2Y_4$ receptor.

The role of P2Y receptors in proximal tubule sodium reabsorption has been postulated [1]. The expression of $P2Y_6$ was shown in *Xenopus* oocytes, in basolateral membrane, and the stimulation of this receptor increased NaCl secretion [41]. Activation of the apical proximal tubule $P2Y_1$ receptor has been associated with a decrease in bicarbonate that involves the NHE3 cotransporter [18]. The results for luminal Ap4A perfusion, which binds to $P2Y_1$ and $P2Y_4$, show a decrease in fractional proximal tubular reabsorption [42]. Our data show that ATP, through PKC-mediated $P2Y_2$ and/or $P2Y_4$ receptor, increases Na^+-ATPase activity in LLC-PK1 cells. Although $P2Y_2$ and $P2Y_4$ receptors are able to increase PKC activity, studies by Rieg et al. [43] on $P2Y_2$ receptor knockout mice infused with

the $P2Y_{2,4}$ agonist, INS45973, indicate that both receptors could have opposite effects on blood pressure and renal sodium excretion. They observed that $P2Y_4$ increased blood pressure and decreased renal sodium excretion. On the other hand, the $P2Y_2$ activation decreased blood pressure and increased renal sodium excretion. Thus, our results indicate that the effect of ATP on Na^+-ATPase activity could be involved in antinatriuresis induced by $P2Y_4$ receptor or a mechanism to counterbalance the natriuretic effect of $P2Y_2$ receptor, promoting fine control of sodium reabsorption in proximal tubule cells.

Acknowledgments

We thank Shanserley Leite do Espírito Santo and Mario Luiz da Silva Bandeira (FAPERJ TCT fellowship) for technical support. This work was supported by grants from Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), Fundação Carlos Chagas Filho de Amparo à Pesquisa do Estado do Rio de Janeiro (FAPERJ), Instituto Nacional de Ciência e Tecnologia para Pesquisa Translacional em Saúde e Ambiente na Região Amazônica (INPeTAm/CNPq), and Instituto Nacional de Ciência e Tecnologia em Biologia Estrutural e Bioimagem (INBEB/CNPq).

References

[1] H.A. Praetorius, J. Leipziger, Annu. Rev. Physiol. 72 (2010) 377–393.
[2] M.P. Abbracchio, G. Burnstock, J.M. Boeynaems, E.A. Barnard, J.L. Boyer, C. Kennedy, G.E. Knight, M. Fumagalli, C. Gachet, K.A. Jacobson, G.A. Weisman, Pharmacol. Rev. 58 (2006) 281–341.
[3] R.M. Vekaria, R.J. Unwin, D.G. Shirley, J. Am. Soc. Nephrol. 17 (2006) 1841–1847.
[4] J. Leipziger, Curr. Opin. Nephrol. Hypertens. 20 (2011) 518–522.
[5] G.G. Yegutkin, Biochim. Biophys. Acta 1783 (5) (2008) 673–694.
[6] V. Vallon, B. Muhlbauer, H. Osswald, Physiol. Rev. 86 (2006) 901–940.
[7] M. Wengert, C. Berto Jr., J. Kaufman, L.R. Leão-Ferreira, R. Paes-de-Carvalho, A.G. Lopes, C. Caruso-Neves, Int. J. Biochem. Cell Biol. 37 (2005) 155–165.
[8] J. Du, L. Zhang, Y. Yang, W. Li, L. Chen, Y. Ge, C. Sun, Y. Zhu, L. Gu, Cell. Physiol. Biochem. 25 (2010) 501–510.
[9] V. Vallon, Am. J. Physiol. Renal Physiol. 294 (1) (2008) F10–F27.
[10] V. Vallon, T. Rieg, Am. J. Physiol. Renal Physiol. 301 (2011) 463–475.
[11] Y. Zhang, R. Listhrop, C.M. Ecelbarger, B.K. Kishore, Am. J. Physiol. Renal Physiol. 300 (2011) F657–F668.
[12] J.D. Stockand, E. Mironova, V. Bugaj, T. Rieg, P.A. Insel, V. Vallon, J. Peti-Peterdi, O. Pochynyuk, J. Am. Soc. Nephrol. 21 (2010) 1903–1911.
[13] R.D. Marques, P.I. de Bruijn, M.V. Sorensen, M. Bleich, H.A. Praetorius, J. Leipziger, Am. J. Physiol. Renal Physiol. 302 (2012) F487–494.
[14] S.S. Wildman, E.S. Kang, B.F. King, Purinergic Signalling 4 (2009) 481–489.
[15] K.M. O'Shaughnessy, F.E. Karet, Annu. Rev. Nutr. 26 (2006) 343–365.
[16] T. Morgan, Hypertens. Res. 26 (2003) 349–354.
[17] A.G. Cabado, F.H. Yu, A. Kapus, G. Lukacs, S. Grinstein, J. Orlowski, J. Biol. Chem. 271 (1996) 3590–3599.
[18] M.A. Bailey, Am. J. Physiol. Renal Physiol. 287 (2004) F789–796.
[19] F. Proverbio, T. Proverbio, R. Marin, Biochim. Biophys. Acta 858 (1986) 202–205.
[20] J.R. Del Castillo, R. Marin, T. Proverbio, F. Proverbio, Biochim. Biophys. Acta 692 (1982) 61–68.
[21] W. Jin, U. Hopfer, Am. J. Physiol. 272 (1997) C1169–C1177.
[22] M.A. Rocafull, F.J. Romero, L.E. Thomas, J.R. del Castillo, Biochim. Biophys. Acta 2011 (1808) 1684–1700.
[23] N. Assaife-Lopes, M. Wengert, A.A. Pinheiro, S.S. Landgraf, R. Paes-de-Carvalho, L.R. Leão-Ferreira, C. Caruso-Neves, Arch. Biochem. Biophys. 486 (2009) 44–50.
[24] O.H. Lowry, N.J. Rosebrough, A.L. Farr, R.J. Randall, J. Biol. Chem. 193 (1951) 265–275.
[25] C. Grubmeyer, H.S. Penefsky, J. Biol. Chem. 256 (1981) 3718–3727.
[26] A.M. De Souza, T.L. de Carvalho, L.D. Lara, E. Gomes-Quintana, A.G. Lopes, C. Caruso-Neves, Biochim. Biophys. Acta 3 (2009) 354–359.
[27] C. Caruso-Neves, A.T. Malaquias, F.F. Loss, V.M. Correa da Costa, V.O. Gomes, A.G. Lopes, Regul. Pept. 115 (2003) 195–201.
[28] H. Towbin, T. Staehelin, J. Gordon, Proc. Natl. Acad. Sci. USA 76 (1979) 4350–4354.
[29] M.A. Bailey, M. Imbert-Teboul, C. Turner, S. Marsy, K. Srai, G. Burnstock, R.J. Unwin, Kidney Int. 58 (2000) 1893–1901.
[30] M.A. Bailey, M. Imbert-Teboul, C. Turner, S.K. Srai, G. Burnstock, R.J. Unwin, J. Am. Soc. Nephrol. 12 (2001) 1640–1647.
[31] K.A. Jacobson, A.A. Ivanov, S. de Castro, T.K. Harden, H. Ko, Purinergic Signalling 5 (2009) 75–89.
[32] M.G. Zizzo, M. Mastropaolo, J. Grählert, F. Mulè, R. Serio, Purinergic Signalling 8 (2012) 275–285.
[33] T. Sezaki, K. Inada, T. Sogabe, K. Kakuda, L. Tomiyama, Y. Matsuno, T. Ichikawa, M. Matsuo, K. Ueda, N. Kioka, PLoS One 7 (2012) e35519.
[34] M.C. Ribeiro, M.S. Costa-Alves, M. Wengert, J.R. Meyer-Fernandes, P. Zancan, C. Caruso-Neves, A.A. Pinheiro, Biochim. Biophys. Acta 2012 (1820) 2030–2036.
[35] E.K. Jackson, R.K. Dubey, Am. J. Physiol. Renal Physiol. 281 (2001) F597–F612.
[36] G. Malnic, H. Enokibara, M.M. Aires, F.L. Vieira, Pflugers Arch. 309 (1969) 21–37.
[37] E.P. Queiroz-Madeira, L.S. Lara, M. Wengert, S.S. Landgraf, J.D. Líbano-Soares, G. Zapata-Sudo, R.T. Sudo, C.M. Takiya, E. Gomes-Quintana, A.G. Lopes, C. Caruso-Neves, Biochim. Biophys. Acta 1798 (2010) 360–366.
[38] L.B. Rangel, C. Caruso-Neves, L.S. Lara, A.G. Lopes, Biochim. Biophys. Acta 1564 (2002) 310–316.
[39] M. Wengert, J. Adao-Novaes, N. Assaife-Lopes, L.R. Leao-Ferreira, C. Caruso-Neves, Arch. Biochem. Biophys. 467 (2007) 261–267.
[40] M.A. Bailey, D.G. Shirley, Purinergic Signalling 5 (2009) 473–480.
[41] M. Köttgen, T. Löffler, C. Jacobi, R. Nitschke, H. Pavenstädt, R. Schreiber, S. Frische, S. Nielsen, J. Leipziger, J. Clin. Invest. 111 (2003) 371–379.
[42] A. Stiepanow-Trzeciak, M. Jankowski, S. Angielski, M. Szczepanska-Konkel, Nephron Physiol. 106 (2007) 13–18.
[43] T. Rieg, M. Gerasimova, J.L. Boyer, P.A. Insel, V. Vallon, Am. J. Physiol. Regul. Integr. Comp. Physiol. 301 (2011) 510–518.