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Short-term regulation of the Cl^-/HCO_3^- exchanger in immortalized SHR proximal tubular epithelial cells

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ABSTRACT

The present study evaluated the activity of Cl^-/HCO_3^- exchanger and the abundance of Slc26a6 in immortalized renal proximal tubular epithelial (PTE) cells from the Wistar–Kyoto rat (WKY) and spontaneously hypertensive rat (SHR) and identified the signaling pathways that regulate the activity of the transporter. The affinity for HCO_3^- was identical in WKY and SHR PTE cells, but V_{max} values (in pH units/min) in SHR PTE cells (0.4016) were significantly higher than in WKY PTE cells (0.2304). The expression of Slc26a6 in SHR PTE cells was sevenfold that in WKY PTE cells. Dibutyryl-cAMP (db-cAMP) or forskolin, which increased endogenous cAMP, phorbol-12,13-dibutyrate (PDBu) and anisomycin, significantly ($P < 0.05$) increased the Cl^-/HCO_3^- exchanger activity in WKY and SHR PTE cells to a similar extent. The stimulatory effects of db-cAMP and forskolin were prevented by the PKA inhibitor H89, but not by chelerythrine. The stimulatory effects of PDBu were prevented by both chelerythrine and SB 203580, but not by H89 or the MEK inhibitor PD 98059. The stimulatory effect of anisomycin was prevented by SB 203580, but not by chelerythrine. Increases in phospho-p38 MAPK by anisomycin were identical in WKY and SHR PTE cells, this being sensitive to SB 203580 but not to chelerythrine. It is concluded that SHR PTE cells, which overexpress the Slc26a6 protein, are endowed with an enhanced activity of the Cl^-/HCO_3^- exchanger. The Cl^-/HCO_3^- exchanger is an effector protein for PKA, PKC and p38 MAPK in both WKY and SHR PTE cells.

1. Introduction

Cl^-/base exchangers belong to a recently discovered gene family of highly versatile anion exchangers, the SLC26 gene family, that transport an expanding number of monovalent and divalent anions, including sulfate (SO_4^{2-}), chloride (Cl^-), iodide (I^-), formate, oxalate, hydroxyl ion (OH^-), and bicarbonate (HCO_3^-) [1]. Putative anion transporter 1 (PAT1 or Slc26a6), which is a member of these highly conserved family of membrane proteins, was shown to be located at the apical membrane of rat and mouse kidney proximal tubules and mediate Cl^-/HCO_3^- and Cl^-/OH^- exchange [2,3]. Functional studies indicate that the main mechanism of Cl^- reabsorption in the kidney proximal tubule is via apical Cl^-/base exchanger that works in parallel with the apical type 3 Na^+/H^+ exchanger (NHE3) and is essential for the Cl^- reabsorption, fluid balance

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and acid–base regulation [4,5]. Actually, approximately 50–70% of the filtered Cl^- is reabsorbed in the proximal tubule [5]. This is in line with observation that the majority of Cl^- and HCO_3^- filtered by kidney are reabsorbed in the proximal tubule. Our group recently demonstrated the presence of an apical Cl^-/HCO_3^- exchanger in immortalized renal proximal tubular epithelial (PTE) cells from the spontaneously hypertensive rat (SHR) and Wistar–Kyoto rat (WKY) [6], which may correspond to the Slc26a6 protein. WKY PTE cells respond to D_1-like dopamine receptor stimulation with inhibition of the apical Cl^-/HCO_3^- exchanger, whereas SHR PTE cells have a defective D_1-like dopamine response [6]. This defective D_1 receptor regulation of the Cl^-/HCO_3^- exchanger in SHR cells [6], is identical to that shown for the NHE3 and Na^+–K^+-ATPase [7–12]. Another difference between WKY and SHR PTE cells concerned the basal activity of the Cl^-/HCO_3^- exchanger, which was greater in the latter than in the former.

The role of protein kinases in the short-term regulation of the Cl^-/HCO_3^- exchanger is still a matter of debate. Some authors suggest that PKC leads to inhibition of the Cl^-/HCO_3^- exchanger [13], whereas others have suggested that regulation of Slc26a6 activity by PKC results from reduced surface expression of the transporter [14]. This contrast with the observations that stimulation of AT_1 receptors and α_1-adrenoceptors in WKY and SHR PTE cells led to increases in the activity of the Cl^-/HCO_3^- exchanger, this being sensitive to inhibition by the PKC inhibitor chelerythrine [15–17]. The immortalized renal PTE cells from WKY and SHRs are well-established models in our laboratories that have been used to evaluate both the diversity and the regulation of electrolyte and amino acid transport systems [6,9–12].

In an attempt to better understand differences in Cl^-/HCO_3^- exchanger activity in hypertension, the present study examined the expression and activity of the Cl^-/HCO_3^- exchanger (Slc26a6) in immortalized renal PTE cells from SHR and WKY and its sensitivity to modulators of a series of transduction pathways commonly used by G protein coupled receptors. It is reported that SHR PTE cells are endowed with an enhanced activity of the Cl^-/HCO_3^- exchanger, characterized by increases in V_{max} values with no changes in the affinity for HCO_3^-. The increase in Cl^-/HCO_3^- exchanger activity in SHR PTE cells was accompanied by overexpression of the Slc26a6 protein. However, the Cl^-/HCO_3^- exchanger in SHR PTE cells responded to PKA, PKC and p38 MAPK stimulation similarly to that observed in WKY PTE cells.

2. Methods and materials

2.1. Cell culture

Immortalized renal PTE cells from 4- to 8-week-old WKY and SHR animals [18] were maintained in a humidified atmosphere of 5% CO_2–95% air at 37 °C. WKY and SHR PTE cells were grown in Dulbecco's modified Eagle's medium nutrient mixture F-12 Ham (Sigma Chemical Company, St. Louis, MO, USA) supplemented with 100 U/ml penicillin G, 0.25 μg/ml amphotericin B, 100 μg/ml streptomycin (Sigma), 4 μg/ml dexamethasone (Sigma), 5 μg/ml transferrin (Sigma), 5 μg/ml insulin (Sigma), 5 ng/ml selenium (Sigma), 10 ng/ml epidermal growth factor (Sigma), 5% fetal bovine serum (Sigma) and 25 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanosulfonic acid (HEPES; Sigma). For subculturing, the cells were dissociated with 0.10% trypsin–EDTA, split 1:8 and subcultured in Costar plates with 21-cm^2 growth areas (Costar, Badhoevedorp, The Netherlands). For pH_i measurement experiments, cells were grown in 96-well plates (Costar) or glass coverslips. For the measurement of Slc26a6 expression the cells were seeded in six-well plastic culture clusters (Costar). The cell medium was changed every 2 days, and the cells reached confluence after 3–5 days of incubation. For approximately 2 h prior to each experiment, the cells were maintained in fetal bovine serum-free medium. Experiments were generally performed 1–2 days after cells reached confluence and 4–5 days after the initial seeding; each cm^2 contained about 50 μg of cell protein.

2.2. pH_i measurements

In intracellular pH measurement experiments, WKY and SHR PTE cells were grown in 96-well plates. Intracellular pH was measured as previously described [11]. At days 4–5 after seeding SHR and WKY PTE cells were incubated at 37 °C for 30 min with 10 μM BCECF-AM. Cells were then washed twice with prewarmed modified Krebs–Hensleit buffer before initiation of the fluorescence recordings. The Krebs medium had the following composition (in mM): 115 NaCl, 25 $NaHCO_3$, 5.4 KCl, 2.8 $CaCl_2$, 1.2 $MgSO_4$, 0.3 NaH_2PO_4, 0.3 KH_2PO_4, 10 HEPES, 5 glucose, pH 7.4 (adjusted with Tris base). Cells were placed in the sample compartment of a dual-scanning microplate spectrofluorometer (Spectramax Gemini XS, Molecular Devices, Sunnyvale, USA), and fluorescence was measured every 17 s alternating between 440 and 490 nm excitation at 535 nm emission, with a cutoff filter of 530 nm. The ratio of intracellular BCECF fluorescence at 490 and 440 nm was converted to pH_i by comparison with values from an intracellular calibration curve using nigericin 10 μM in a high-K^+ solution (in mM: 15 NaCl, 130 KCl, 0.3 KH_2PO_4, 0.3 NaH_2PO_4, 5 glucose, 1.2 $MgSO_4$, 2.8 $CaCl_2$ and 10 HEPES) with pH ranging from 6.6 to 7.8 [19].

2.3. Cl^-/HCO_3^- exchanger activity

The Na^+-independent HCO_3^- transport system activity was assayed as the initial rate of pH_i recovery after an alkaline load (CO_2/HCO_3 removal), in the absence of Na^+, as previously described [6]. Briefly, cells were loaded in serum-free medium with 10 μM BCECF-AM, the membrane-permeant acetoxymethyl ester derivative of 2′,7′-bis (carboxyethyl)-5,6-carboxyfluorescein (BCECF) for 30 min at 37 °C in 5% CO_2–95% air atmosphere. The cells were washed free of dye and loaded with Krebs–Hensleit solution (25 mM $NaHCO_3$) for 25 min (phase 1). Then, extracellular solution was replaced by a Krebs–Hensleit $NaHCO_3$-free solution (phase 2; 10 min). In the $NaHCO_3$-free bathing solution, $NaHCO_3$ was replaced by an equimolar concentration of choline. In experiments intended to evaluate the K_m for HCO_3, $NaHCO_3$ in the Krebs–Hensleit solution (phase 1, 25 min) was replaced by an equimolar concentration of sodium gluconate. The test compounds were added to the extracellular fluid 40 min before starting the

bicarbonate-dependent pH_i recovery. The concentrations of kinase activators/inhibitors used in this study were based on the efficacy data for these compounds described in the literature [6,20,21]. Cells were placed in the sample compartment of a dual-scanning microplate spectrofluorometer (Spectramax Gemini, Molecular Devices, Sunnyvale, USA) and fluorescence monitored every 17 s alternating between 440 and 490 nm excitation at 535 nm of emission, with a cutoff filter of 530 nm.

2.4. Measurement of cyclic AMP

Cyclic AMP (cAMP) was determined with an enzyme immunoassay kit (GE Healthcare) as previously described [22]. Cells were preincubated for 15 min at 37 °C in Hanks' medium ((in mM) 137 NaCl, 5 KCl, 0.8 $MgSO_4$, 0.33 Na_2HPO_4, 0.44 KH_2PO_4, 0.25 $CaCl_2$, 1.0 $MgCl_2$, 0.15 Tris–HCl, and 1.0 Na^+ butyrate, pH 7.4) containing 100 μM IBMX, a phosphodiesterase inhibitor. Cells were then incubated for 15 min with test compounds. At the end of the experiment, the reaction was stopped by the addition of 0.1 M HCl. Aliquots were taken for the measurement of intracellular cAMP content.

2.5. Immunobloting

WKY and SHR PTE cells cultured to 90% of confluence were washed twice with PBS and total cell protein extracted for Slc26a6 and phospho-p38 MAPK detection. Briefly, to obtain total cell extract, cells were lysed by brief sonication (15 s) in RIPA buffer with protease inhibitors (150 mM NaCl, 50 mM Tris–HCl pH 7.4, 5 mM EDTA, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 100 μg/ml PMSF, aprotinin and leupeptin 2 μg/ml each) and incubated on ice for 1 h. Renal cortical membranes were washed with PBS and then lysed in RIPA buffer, as above. After centrifugation (16,000 × *g*, 30 min, 4 °C), the supernatant was collected and protein concentration determined using a protein assay kit (Bio-Rad Laboratories, Hercules, CA) with bovine serum albumin as standard. Twenty micrograms of protein were mixed in 6× sample buffer (0.35 M Tris–HCl, 4% SDS, 30% glycerol, 9.3% DTT, pH 6.8, 0.01% bromophenol blue) and boiled for 5 min. Proteins were subjected to SDS-PAGE (10% SDS-polyacrylamide gel) and electrotransfered onto nitrocellulose membranes. The transblot sheets were blocked with 5% of non-fat dry milk in Tris–HCl 25 mM pH 7.5, NaCl 150 mM and 0.1% Tween 20, overnight at 4 °C. Then, the membranes were incubated with rabbit polyclonal anti-Slc26a6 antibody (1:1000; [23]), the anti-β-actin primary antibody (1:10,000; Lab Vision Corporation) and the anti-phospho-p38 MAPK primary antibody (1:1000; Cell Signaling Technology™, Beverly, MA) in 5% non-fat dry milk in PBS-T overnight at 4 °C. The immunoblots against Slc26a6, phospho-p38 MAPK and β-actin were subsequently washed and incubated with fluorescent labeled goat anti-rabbit (1:20,000; IRDye™ 800, Rockland) or a fluorescent labeled goat anti-mouse secondary antibody (1:20,000; AlexaFluor 680, Molecular Probes), respectively, for 60 min at room temperature and protected from light. The membrane was washed and imaged by scanning at both 700 and 800 nm, with an Odyssey Infrared Imaging System (LI-COR Biosciences).

2.6. Drugs

Chelerythrine chloride, dibutyryl-cAMP (db-cAMP), forskolin, parathyroid hormone, H89, phorbol-12,13-dibutyrate (PDBu) and 4α-phorbol 12,13-didecanoate were purchased from Sigma Chemical Company, St. Louis, MO, USA. Anisomycin, PD 98059 and SB 203580 were obtained from Research Biochemicals International (Natick, USA). Acetoxymethyl ester of 2′,7′-bis(carboxyethyl)-5(6)-carboxyfluorescein (BCECF-AM) and nigericin were obtained from Molecular Probes (Eugene, OR).

2.7. Data analysis

Geometric means are given with 95% confidence limits and arithmetic means are given with S.E.M. Statistical analysis was performed by One-way analysis of variance (ANOVA) followed by the Newman–Keuls test for multiple comparisons. A *P*-value less than 0.05 were assumed to denote a significant difference.

3. Results

The activity of the Cl^-/HCO_3^- exchanger was assayed as the initial rate of pH_i recovery after an alkaline load (CO_2/HCO_3 removal) in the absence of sodium to avoid the contribution of other transporters such as the NHE3. In a HCO_3-containing medium, removal of CO_2/HCO_3 caused an initial cell alkalinization, as a result of CO_2 loss from the cell with subsequent return of pH_i towards basal values (Fig. 1A). The Na^+-independent Cl^-/HCO_3^- exchanger (1 external Cl^- exchanged for 1 internal HCO_3^-) mediated the pH_i recovery process after the alkalinization. As shown in Fig. 1A, the HCO_3^--dependent recovery of pH_i in SHR PTE cells was steeper than that observed in WKY PTE cells. The HCO_3^--dependent pH_i recovery rates (in pH units/s) during the linear phase of pH_i recovery after intracellular alkalinization were greater in SHR cells than in WKY cells (0.2523 ± 0.01 versus 0.1643 ± 0.01 delta pH units/min, $n = 32$). The relationship between Cl^-/HCO_3^- exchanger activity and intracellular HCO_3^- was measured after loading the cells with increasing concentrations of HCO_3^- (0–25 mM) ($NaHCO_3$ was replaced by equimolar concentration of sodium gluconate). In both WKY and SHR PTE cells, increases in extracellular HCO_3^- increased the activity of the Cl^-/HCO_3^- exchanger (Fig. 1B). The affinity for HCO_3^- was identical in WKY and SHR PTE cells, but V_{max} values in SHR PTE cells were significantly higher than in WKY PTE cells (Table 1).

The expression of Slc26a6 was evaluated in immortalized renal PTE cells from SHR and WKY and in kidney from WKY and SHR rats (12 weeks). Immunoblot analysis showed that the polyclonal anti-Slc26a6 antibody stained only one band in immortalized renal PTE cells from WKY and SHR and in renal cortical membranes from WKY and SHR rats. The bands were not seen upon pre-adsorption of the antibody with the immunizing peptide that was used to produce the rabbit polyclonal anti-Slc26a6 (data not shown). As shown in Fig. 2, the level of expression of Slc26a6 in SHR PTE cells and in SHR renal cortical membranes was sevenfold that in WKY PTE cells and WKY renal cortical membranes. This result fits well the

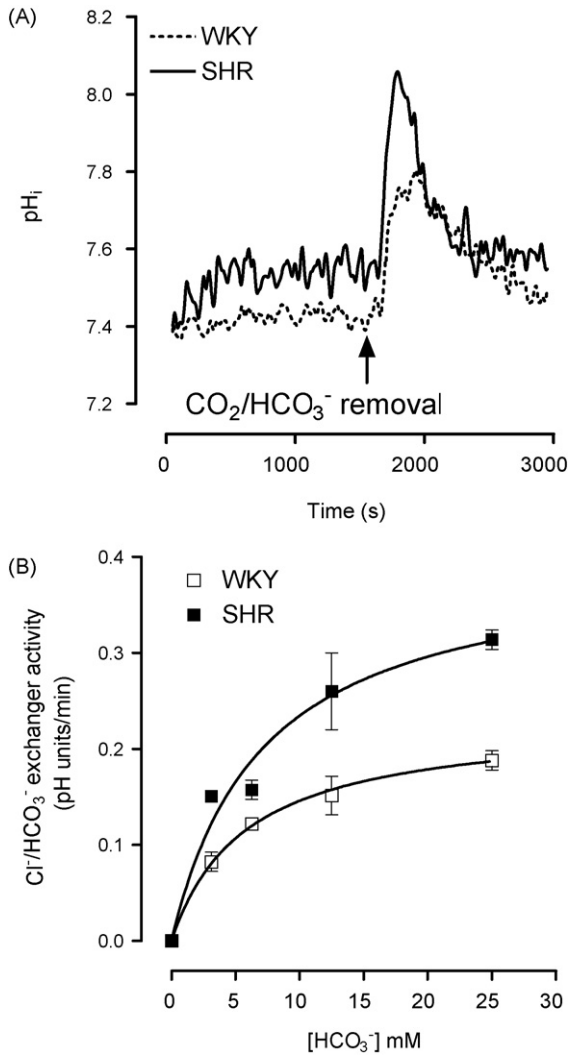

Fig. 1 – (A) Assessment of intracellular pH before and after an alkaline load (CO_2/HCO_3^- removal) in perfused SHR and WKY cells. Traces represent means of three experiments per group. (B) Cl^-/HCO_3^- exchanger activity as a function of extracellular HCO_3^- concentration in immortalized WKY and SHR PTE cells. Symbols represent mean of seven independent determinations; vertical lines show S.E.M.

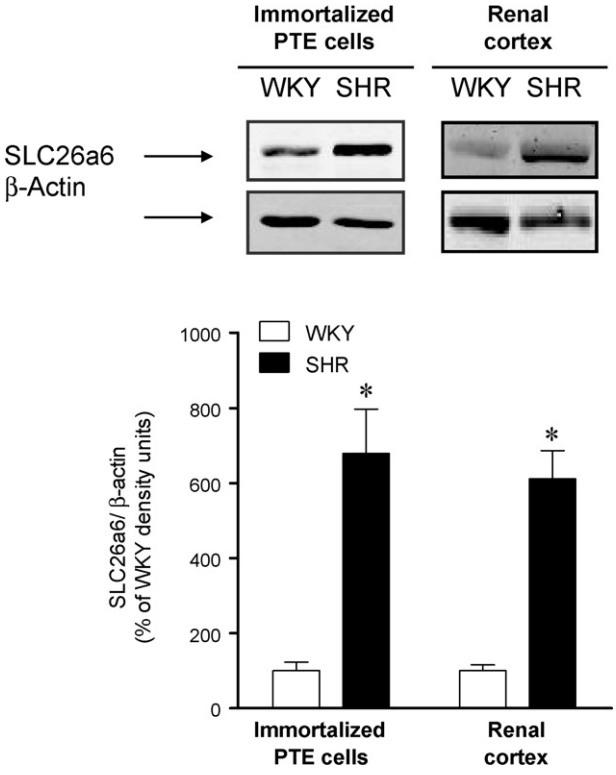

Fig. 2 – Expression of Slc26a6 and β-actin in immortalized WKY and SHR PTE cells and in WKY and SHR (12 weeks) renal cortical membranes. Representative immunoblots are depicted on top of the bar graphs. Columns represent mean of five independent immunoblots; vertical lines show S.E.M. Significantly different from values in WKY (*$P < 0.05$); Slc26a6 ~70 kDa; β-actin ~40 kDa.

view that SHR PTE cells have an increased activity of the apical Cl^-/HCO_3^- exchanger that is mediated by the Slc26a6 transporter.

To evaluate whether Cl^-/HCO_3^- exchanger is an effector protein for PKA, PKC and p38 MAPK in WKY and SHR PTE cells, the effects of db-cAMP and forskolin, phorbol-12,13-dibutyrate (PDBu), an activator of classical and novel PKCs, and anisomycin, respectively, were examined. Activation of PKA with db-cAMP or forskolin, which increased endogenous cAMP accumulation (from 1461.2 ± 635.9 to 3741.6 ± 614.3 fmol/well in WKY PTE cells and from 945.5 ± 353.2 to 3736.8 ± 582.6 fmol/well in SHR PTE cells), activation of PKC

Table 1 – Kinetic parameters (K_m, mM; V_{max} pH units/min) for Cl^-/HCO_3^- exchanger activity in immortalized WKY and SHR PTE cells

Treatment	K_m (mM)		V_{max} (pH units/min)	
	WKY	SHR	WKY	SHR
Control	5.8 ± 0.1	7.1 ± 0.6	0.2304 ± 0.0014	0.4016 ± 0.0010 *
Anisomycin	6.4 ± 0.4	6.0 ± 0.2	0.2662 ± 0.0055 #	0.5008 ± 0.0061 #
Forskolin	4.8 ± 0.4	6.3 ± 0.3	0.2643 ± 0.0064 #	0.4709 ± 0.0074 #

Values are mean ± S.E.M. of 5–8 experiments per group. Significantly different from WKY values (*$P < 0.05$) and from corresponding control values (#$P < 0.05$).

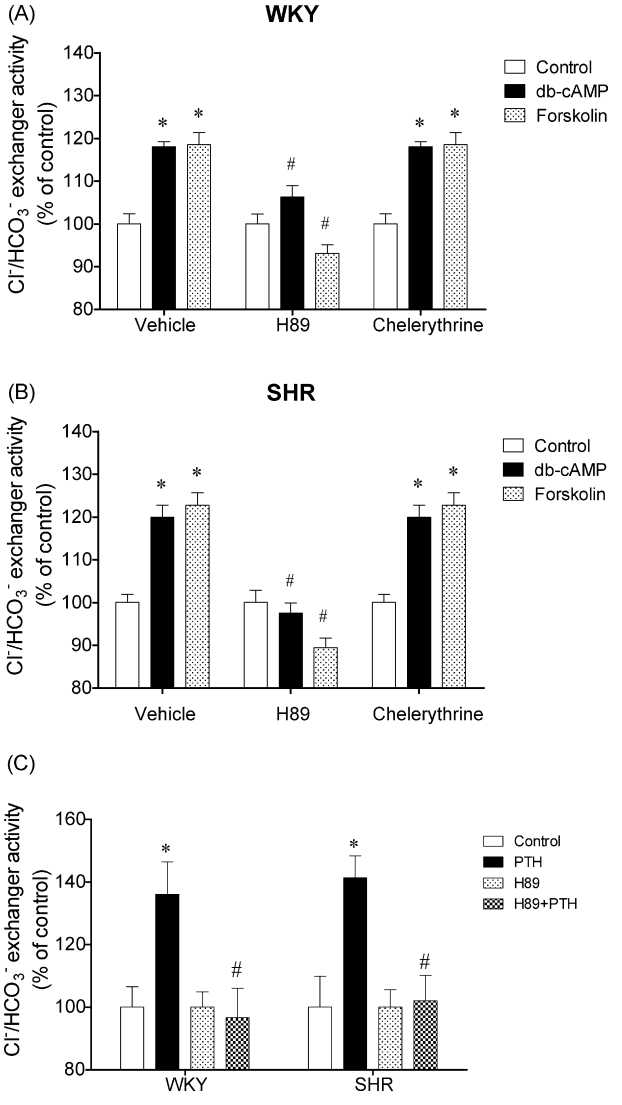

Fig. 3 – Effect of dibutyryl cAMP (db-cAMP, 500 μM) and forskolin (30 μM) for 40 min on Cl^-/HCO_3^- exchanger activity, in the absence and the presence of H89 (10 μM) or chelerythrine (1 μM) in (A) WKY and (B) SHR PTE cells. Each column represents the mean of 6–8 experiments per group; vertical lines indicate S.E.M. Significantly different from corresponding control values (*$P < 0.05$) and values for db-cAMP or forskolin alone (#$P < 0.05$). (C) Effect of PTH (10 nM) for 40 min on Cl^-/HCO_3^- exchanger activity, in the absence and the presence of H89 (10 μM) in WKY and SHR PTE cells. Each column represents the mean of 6–8 experiments per group; vertical lines indicate S.E.M. Significantly different from corresponding control values (*$P < 0.05$) and values for PTH alone (#$P < 0.05$).

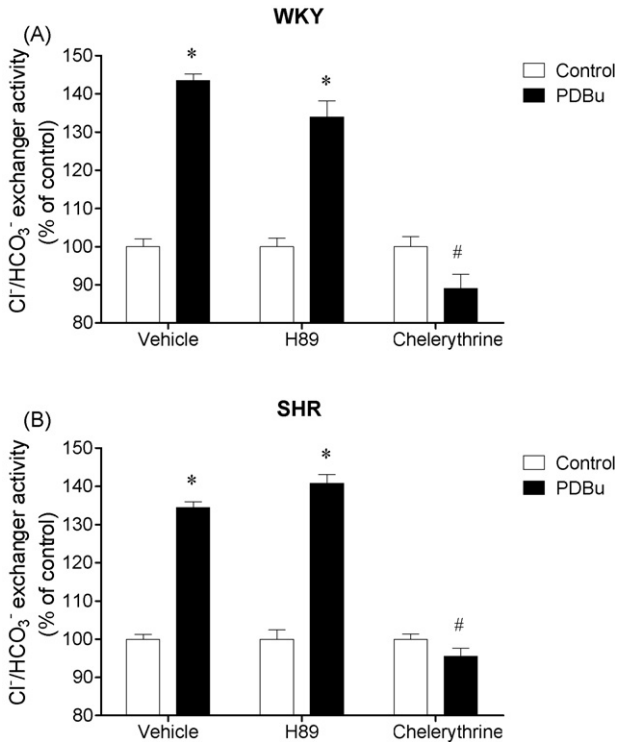

Fig. 4 – Effect of phorbol-12,13-dibutyrate (PDBu, 0.1 μM) for 40 min on Cl^-/HCO_3^- exchanger activity, in the absence and the presence of H89 (10 μM) or chelerythrine (1 μM) in (A) WKY and (B) SHR PTE cells. Each column represents the mean of 6–8 experiments per group; vertical lines indicate S.E.M. Significantly different from corresponding control values (*$P < 0.05$) and values for PDBu alone (#$P < 0.05$).

with PDBu and activation of p38 MAPK with anisomycin, all resulted in increases in Cl^-/HCO_3^- exchanger activity in WKY and SHR PTE cells. Despite differences in Cl^-/HCO_3^- exchanger activity between WKY and SHR PTE cells, the stimulatory effects observed after PKA, PKC and p38 MAPK activation in WKY PTE cells were of identical magnitude to those obtained in SHR PTE cells (Figs. 3–5). The inactive phorbol ester 4α-phorbol 12,13-didecanoate (1 μM) did not affect the Cl^-/HCO_3^- exchanger activity in WKY and SHR PTE cells (data not shown).

As shown in Fig. 3A and B, the stimulatory effects of db-cAMP (500 μM) and forskolin (30 μM) were prevented by the PKA inhibitor H89 (10 μM), but not by the PKC inhibitor chelerythrine (1 μM). In addition to PKA activators, it was also evaluated the activation of PKA via physiological ligand, namely the parathyroid hormone (PTH). As shown in Fig. 3C, PTH (10 nM) stimulated Cl^-/HCO_3^- exchanger activity in WKY and SHR PTE cells, this being prevented by H89 (10 μM). Because interactions between PKA and PKC transduction pathways have been described [10,24,25], we tested whether the PKA and PKC inhibitors interfered with stimulatory effect of PDBu (0.1 μM). As shown in Fig. 4, the stimulatory effects of PDBu were prevented by the PKC inhibitor chelerythrine (1 μM), but not by the PKA inhibitor H89 (10 μM). Chelerythrine and H89 alone had no effect upon Cl^-/HCO_3^- exchanger basal activity in WKY and SHR cells (Table 2). The PKA and PKC inhibitors had not effect on the basal Cl^-/HCO_3^- exchanger activity (in pH units/min) in WKY and SHR cells, as indicated below: WKY cells, vehicle = 0.1617 ± 0.01, H89 = 0.1824 ± 0.01, chelerythrine = 0.1454 ± 0.01; SHR cells, vehicle = 0.2985 ± 0.01, H89 = 0.2942 ± 0.03, and chelerythrine = 0.2911 ± 0.02.

Because interactions between PKC and the MEK and p38 MAPK transduction pathways have been described [26–28], we

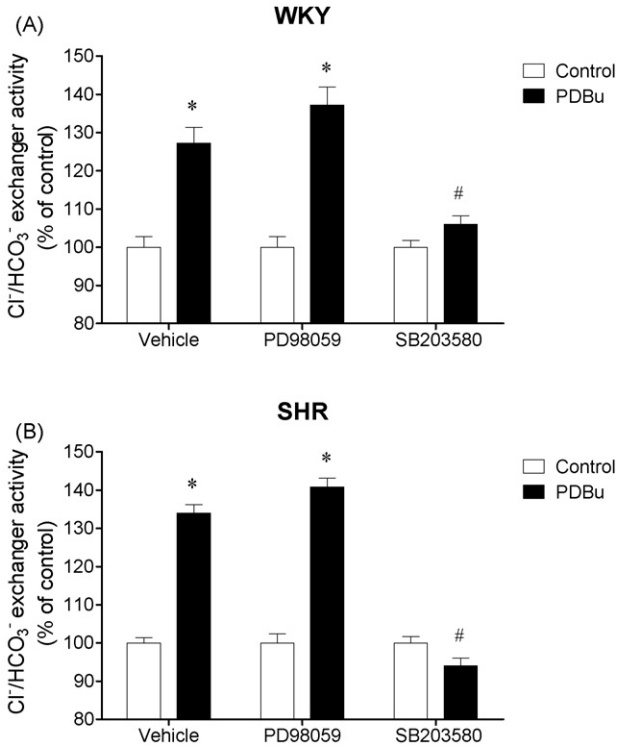

Fig. 5 – Effect of phorbol-12,13-dibutyrate (PDBu, 0.1 μM) for 40 min on Cl^-/HCO_3^- exchanger activity in (A) WKY and (B) SHR PTE cells in the absence and the presence of SB 203580 (10 μM) or PD 98059 (10 μM). Each column represents the mean of 7–15 experiments per group; vertical lines indicate S.E.M. Significantly different from corresponding control values (*P < 0.05) and values for PDBu alone (#P < 0.05).

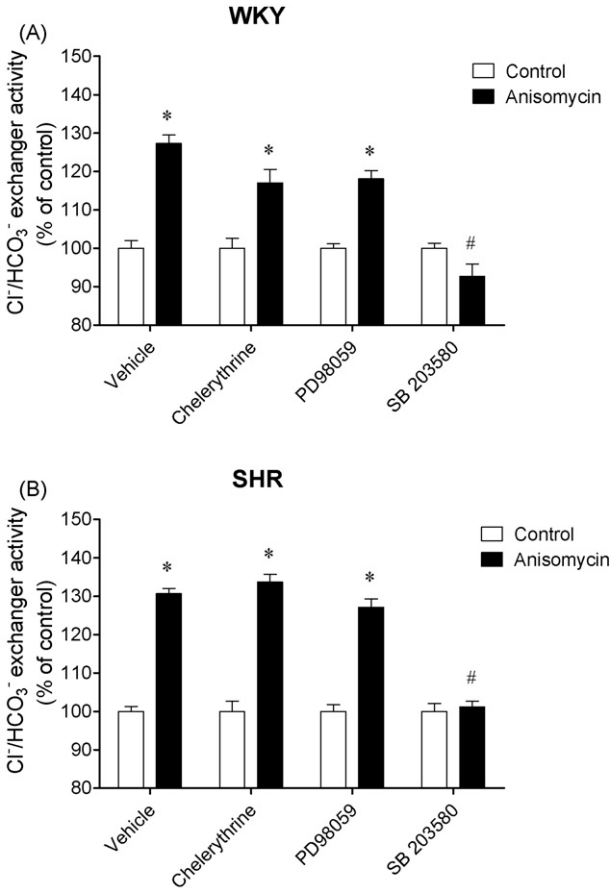

Fig. 6 – Effect of anisomycin (0.1 μM) for 40 min on Cl^-/HCO_3^- exchanger activity in (A) WKY and (B) SHR PTE cells in the absence and the presence of chelerythrine (1 μM), PD 98059 or (10 μM) SB 203580 (10 μM). Each column represents the mean of 7–15 experiments per group; vertical lines indicate S.E.M. Significantly different from corresponding control values (*P < 0.05) and values for anisomycin alone (#P < 0.05).

tested whether the MEK 1 and p38 MAPK inhibitors interfered with stimulatory effect of anisomycin and PDBu. As shown in Fig. 5, the stimulatory effects of PDBu (0.1 μM) upon the Cl^-/HCO_3^- exchanger activity in WKY and SHR PTE cells was not affected by the MEK 1 inhibitor PD 98059 (10 μM), but was prevented by the p38 MAPK inhibitor SB 203580 (10 μM). However, the stimulatory effect of anisomycin (0.1 μM) upon the Cl^-/HCO_3^- exchanger activity in WKY and SHR PTE cells was prevented by SB 203580 (10 μM), but not by chelerythrine (1 μM) and PD 98059 (10 μM) (Fig. 6). PD 98059 and SB 203580 alone had no effect upon Cl^-/HCO_3^- exchanger basal activity in WKY and SHR cells (Table 2). The absolute control values for Cl^-/HCO_3^- exchanger activity in pH units/min in WKY and SHR cells were as follows: WKY cells, vehicle = 0.1617 ± 0.01, PD 98059 = 0.1501 ± 0.01, SB 203580 = 0.1659 ± 0.01; SHR cells, vehicle = 0.2985 ± 0.01, PD 98059 = 0.3117 ± 0.02, and SB 203580 = 0.2863 ± 0.02.

Table 2 – Cl^-/HCO_3^- exchanger activity in WKY and SHR cells in the absence and in the presence of H89 (10 μM), chelerythrine (1 μM), PD 98059 (10 μM) and SB 203580 (10 μM)

Treatment	Cl^-/HCO_3^- exchanger activity (% of control)	
	WKY	SHR
Control	100.0 ± 9.0	100.0 ± 6.9
H89	109.9 ± 7.5	98.5 ± 9.2
Chelerythrine	95.3 ± 5.9	102.6 ± 6.9
PD 98059	93.9 ± 9.2	104.2 ± 5.1
SB 203580	104.4 ± 8.9	90.2 ± 6.1

Values are mean ± S.E.M. of 6–8 experiments per group.

The results described above suggest some level of interaction between PKC and p38 MAPK on the regulation of the Cl^-/HCO_3^- exchanger, as evidenced by finding that the stimulatory effects of PDBu upon the Cl^-/HCO_3^- exchanger activity were prevented by both the PKC inhibitor chelerythrine and p38 MAPK inhibitor SB 203580. To clarify the type of interaction and the sequence of events between PKC and p38 MAPK, phospho-p38 MAPK activation was evaluated in WKY and SHR PTE cells exposed to anisomycin or PDBu for 40 min. Treatment with anisomycin (0.1 μM) markedly resulted in evident activation of phospho-p38 MAPK in WKY and SHR PTE cells, this being sensitive to SB 203580 (10 μM), but not to

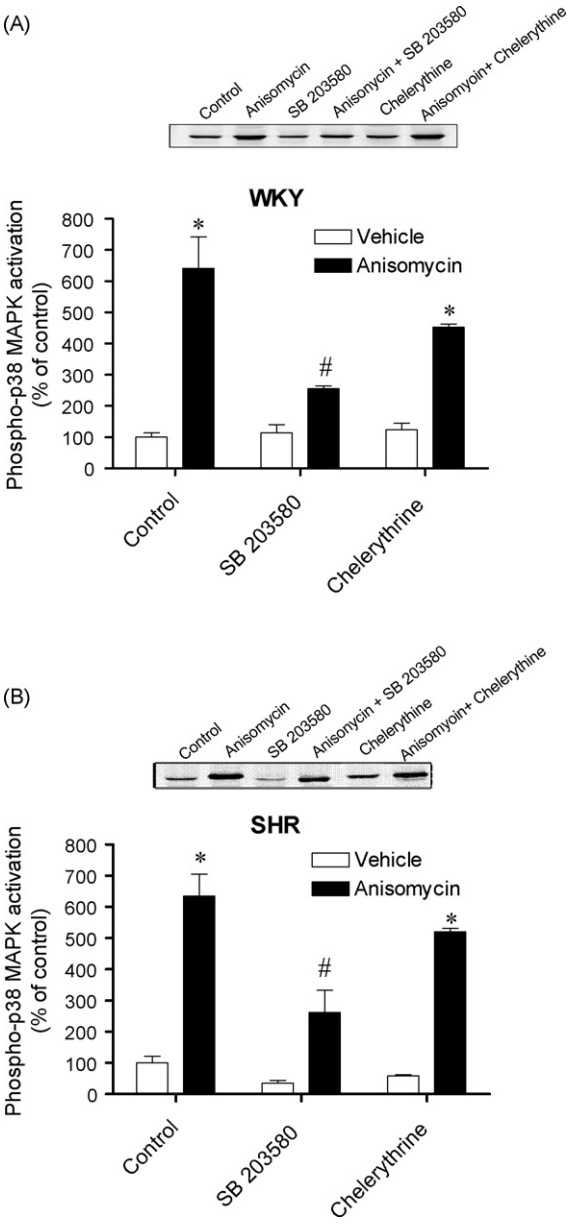

Fig. 7 – Effect of anisomycin (0.1 μM) for 40 min on phospho-p38 MAPK expression in (A) WKY and (B) SHR PTE cells in the absence and the presence of SB 203580 (10 μM) and chelerythrine (1 μM). Each column represents the mean of 4 experiments per group; vertical lines indicate S.E.M. Significantly different from corresponding control values (*P < 0.05) and values for anisomycin alone (#P < 0.05).

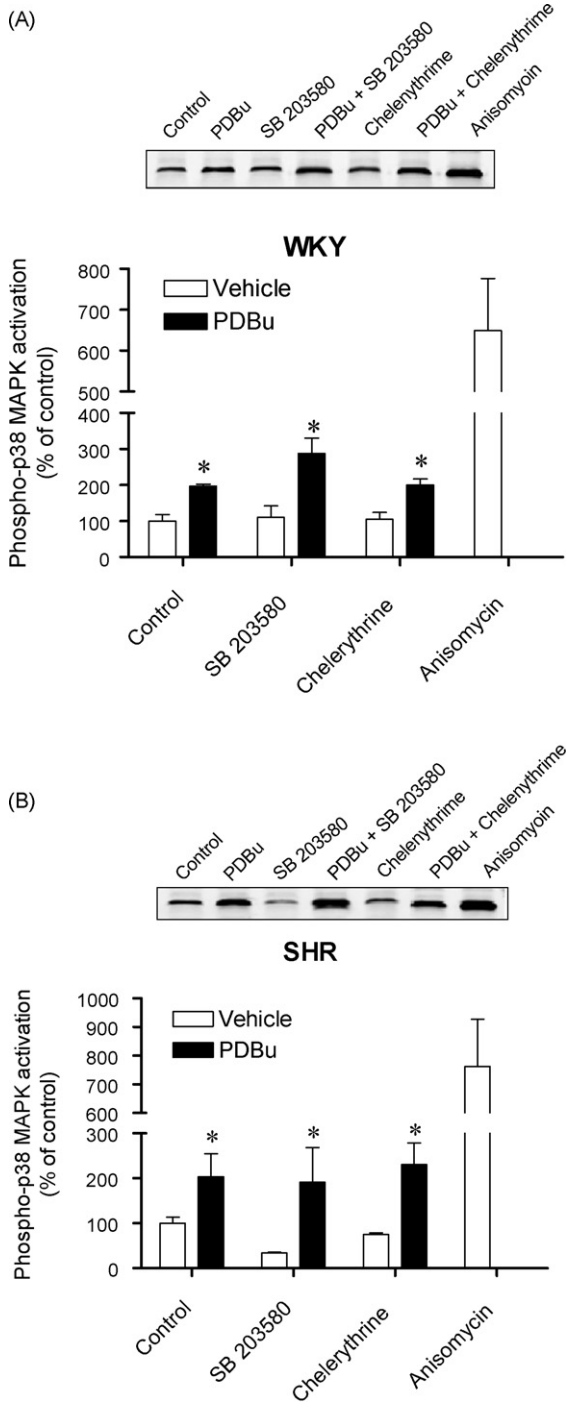

Fig. 8 – Effect of PDBu (0.1 μM) for 40 min on phospho-p38 MAPK expression in (A) WKY and (B) SHR PTE cells in the absence and in the presence of SB 203580 (10 μM) and chelerythrine (1 μM). Each column represents the mean of 4 experiments per group; vertical lines indicate S.E.M. Significantly different from corresponding control values (*P < 0.05).

chelerythrine (1 μM) (Fig. 7). Treatment of WKY and SHR PTE cells with PDBu (0.1 μM) increased phospho-p38 MAPK, but the magnitude of this increases was much lower than that obtained with anisomycin. The increases in phospho-p38 MAPK by PDBu (0.1 μM) were identical in WKY and SHR PTE cells, this being insensitive to chelerythrine and SB 203580 (Fig. 8). In addition, the effect of PDBu plus anisomycin upon the $Cl^-/HCO3^-$ exchanger activity in both cell lines was also tested. The stimulatory effect of PDBu plus anisomycin upon the Cl^-/HCO_3^- exchanger was of equal magnitude as when PDBu and anisomycin were tested separately, in both WKY and SHR cells (Table 3).

In order to better understand the mechanism of PKA and p38 MAPK-induced stimulation of Cl^-/HCO_3^- exchanger

Table 3 – Cl^-/HCO_3^- exchanger activity in WKY and SHR cells in the absence and in the presence of PDBu (0.1 μM), anisomycin (0.1 μM), PDBu and anisomycin

Treatment	Cl^-/HCO_3^- exchanger activity (% of control)	
	WKY	SHR
Control	100.0 ± 3.2	100.0 ± 6.9
PDBu	127.4 ± 6.7	141.2 ± 7.0
Anisomycin	134.1 ± 7.5	124.1 ± 5.2
PDBu + anisomycin	134.0 ± 9.5	139.3 ± 8.9

Values are mean ± S.E.M. of 5–7 experiments per group.

activity, it was evaluated their role on the kinetic parameters of the exchanger. Activation of PKA with forskolin and p38 MAPK with anisomycin resulted in an increase of V_{max} values, comparatively with the control situation, in both WKY and SHR PTE cells. K_m values were not changed in the presence of forskolin or anisomycin in WKY and SHR PTE cells (Table 1).

4. Discussion

The present study was designed to evaluate the effects of PKA, PKC and p38 MAPK activators on the activity of the Cl^-/HCO_3^- exchanger in immortalized renal PTE cells from WKY and SHR. Some of the SLC26 family members have emerged as candidates to mediate proximal tubule Cl^-/HCO_3^- exchange, like slc26a4 and Slc26a6. Although slc26a4 have been described in the kidney, its expression is not detected in the proximal tubule and there is no change in transtubular NaCl absorption in slc26a4 null mice [29]. In contrast, Slc26a6 is the primary candidate for the apical Cl^-/base exchanger of brush border membranes in the renal proximal tubule and might encode the apical Cl^- entry site involved in NaCl absorption, since it is expressed at the apical membrane of proximal tubule cells and appears to mediate the multiple modes of anion exchange that have been implicated in this process [1]. Though there is no specific assay for Cl^-/HCO_3^- exchange-mediated activity, several findings strongly suggest that pH_i recovery after removal of CO_2/HCO_3^- in the absence of Na^+ reflects the activity of the Cl^-/HCO_3^- exchanger. The pH_i recovery after removal of CO_2/HCO_3^- in the absence of Na^+ is markedly dependent on Cl^- (pH_i recovery reduction: 99.5% and 83.3% in SHR and WKY cells, respectively), which clearly excludes the participation of the NHE and the Na^+–HCO_3^- co-transporter (NBC) in the pH_i recovery process [6]. Moreover, the Cl^-/HCO_3^- exchanger activity was exclusively observed on the apical side of the cells [6], which excludes the contribution of the NBC that is expressed in the basolateral side of the cell [20]. The pH_i recovery in these experimental conditions is strongly inhibited by DIDS [6,23], which fits well the view that NHE does not participate in the pH_i recovery after the alkaline load.

Previous work using transfected HEK293 cells suggested that PKC inhibition of Cl^-/HCO_3^- exchange mediated by human Slc26a6 was related to phosphorylation at a specific site that disrupts binding of Slc26a6 to carbonic anhydrase II, thereby disrupting a HCO_3 transport metabolic [13]. In another study, using *Xenopus* oocytes that mutation of the corresponding phosphorylation site of mouse Slc26a6 had no effect on inhibition in response to PKC activation [14]. These authors demonstrated that regulation of Slc26a6 activity by PKC results from reduced surface expression of the transporter, thereby inhibiting all tested modes of transport [14]. It was suggested of importance in future studies to determine the regulation of Slc26a6 under physiological conditions in native tissues. To our knowledge, the present study is the first systematic evaluation of the short-term regulation of the Cl^-/HCO_3^- exchanger in renal tubular epithelial cells [30]. Discrepancies on the role of PKC in regulating the Cl^-/HCO_3^- exchanger described in the present study and that in the study of Hassan et al. [14], may relate to differences on the type of cells (renal tubular epithelial cells versus *Xenopus* oocytes) or differences on the behaviour of the native protein versus the heterologously expressed protein. It should be underlined, however, that in both WKY and SHR PTE cells the stimulation of AT_1 receptors and α_1-adrenoceptors leads to increases in the activity of the Cl^-/HCO_3^- exchanger, this being abolished by the PKC inhibitor chelerythrine [15–17]. The PKA and PKC inhibitors had not effect on the basal Cl^-/HCO_3^- exchanger activity in WKY and SHR cells, which suggest that under basal physiological conditions the Cl^-/HCO_3^- exchanger is not under the tonic influence of PKA or PKC. Similar observations were reported on the regulation of the organic anion transport driven by the organic anion transporter 3 (OAT3) [31,32].

The effect of db-cAMP and forskolin on Cl^-/HCO_3^- exchanger activity in WKY and SHR PTE cells was abolished by H89 (PKA inhibitor), but not by chelerythrine (PKC inhibitor). On the other hand, stimulation of the exchanger activity in WKY and SHR PTE cells by PDBu was prevented by chelerythrine but not by H89. Taken together, these results indicate that cAMP-induced stimulation of the Cl^-/HCO_3^- exchanger activity occurs through the activation of PKA, whereas the PDBu-induced stimulation of the Cl^-/HCO_3^- exchanger activity occurs through the activation of PKC, these two events being independent of which other. This fits well the view that the Cl^-/HCO_3^- exchanger is an effector protein for both PKA and PKC. This is an interesting finding since several studies have suggested a close interaction between PKA and PKC, namely concerning the regulation of renal tubular Na^+ transporters [10,24,25]. However, it should be underscored that PKA-mediated inhibition of NHE3 in rat renal proximal tubular cells proceeds in a manner completely independent of PKC activation [10]. This is an interesting point since major mechanisms intervening in renal proximal tubular NaCl absorption, intracellular pH and cell volume regulation, occur through the concerted action of the Cl^-/HCO_3^- and Na^+/H^+ exchangers. It is well established that PTH stimulates adenylyl cyclase [33,34]. PTH enhanced Cl^-/HCO_3^- exchanger activity was abolished by H89, indicating that the regulatory mechanism presented here occur in physiological conditions.

Downstream to PKC activation, other mechanisms involved in signal transduction coupled to G protein coupled receptors include activation of both the extracellular signal-regulated kinase (ERK) and p38 MAPK pathways [26–28]. We examined some of these signaling pathways in WKY and SHR PTE cells using specific inhibitors. To evaluate the contribution of MAPK in the regulation of Cl^-/HCO_3^- exchanger activity specific MEK and p38 MAPK inhibitors were used. The finding that stimulation of Cl^-/HCO_3^- exchanger activity induced by

PDBu and anisomycin in WKY and SHR PTE cells was insensitive to PD 98059 excludes the involvement of MEK 1. The observation that PDBu, an activator of PKC, and anisomycin, an activator of p38 MAPK, stimulated Cl^-/HCO_3^- exchanger activity in both WKY and SHR PTE cells, in a SB 203580-sensitive manner, would support the view that some sort of interaction between PKC and p38 MAPK was taking place in events in which the Cl^-/HCO_3^- exchanger is the effector protein. However, the observation that the SB 203580-sensitive anisomycin-induced stimulation was insensitive to chelerythrine would suggest that p38 MAPK was an event downstream in the signaling pathway. In contrast, the observation that the chelerythrine-sensitive PDBu-induced stimulation was sensitive to SB 203580 led us to hypothesize that PKC activation was an event upstream p38 MAPK activation, as has been reported with different stimuli [21,35,36]. Furthermore, no additive effects between PDBu and anisomycin were observed when cells were exposed at the same time to PKC and p38 MAPK activators, suggesting that PKC and p38 MAPK are in the same pathway. However, the observation that increases in phospho-p38 MAPK by PDBu were insensitive to chelerythrine and SB 203580 may suggest that the SB 203580-sensitive PDBu-induced stimulation of the Cl^-/HCO_3^- exchanger might result from unspecific or indirect effects upon p38 MAPK. By contrast, the SB 203580-sensitive anisomycin-induced stimulation of the Cl^-/HCO_3^- exchanger is most likely the result of p38 MAPK activation. This fits well the observation that activation of phospho-p38 MAPK in WKY and SHR PTE cells by anisomycin was sensitive to SB 203580, but not to chelerythrine. On the other hand, the finding that the PDBu-induced stimulation of the Cl^-/HCO_3^- exchanger was sensitive to chelerythrine favours the view that the Cl^-/HCO_3^- exchanger is an effector protein for PKC and does not reflect the unspecific effects of PDBu upon p38 MAPK.

The data reported here shows that SHR PTE cells over-expressed the Cl^-/HCO_3^- exchanger Slc26a6 (sevenfold that WKY cells). Likewise, in renal cortical membranes isolated from the SHR the level of expression of the Slc26a6 was sevenfold that in renal cortical membranes from the WKY, indicating that differences in Scl26a6 protein expression may be genetically determined, may have a role in hypertension, and immortalized proximal tubular epithelial cells maintain the characteristics of the original tissue/organ. On the other hand, SHR PTE cells were endowed with an enhanced Cl^-/HCO_3^- exchanger activity (twofold) when compared with WKY PTE cells. Since expression of Slc26a6 was performed with total cell proteins, it is possible that a sevenfold difference in SHR cells Slc26a6 protein expression only translated into about twofold increase in Cl^-/HCO_3^- exchanger activity due to differences in trafficking of Slc26a6 to the cell membrane. However, despite differences in Cl^-/HCO_3^- exchanger activity the induced stimulation of the PKA, PKC and p38 MAPK activators on the activity of the Cl^-/HCO_3^- exchanger in immortalized renal SHR PTE cells was identical to that observed in WKY PTE cells. It is suggested that a possible mechanism for the PKA, PKC and p38 MAPK stimulation of the Cl^-/HCO_3^- exchanger may involve the movement of the exchangers from an intracellular compartment to the apical cell membrane, playing the kinases an important role enhancing the trafficking of the exchangers. The consequence is an enhanced Cl^-/HCO_3^- exchanger activity upon PKA, PKC and p38 MAPK activation. The fact that activation of PKA and p38 MAPK resulted in increases of V_{max} values supports this view.

It is concluded that SHR PTE cells are endowed with an enhanced activity of the Cl^-/HCO_3^- exchanger, characterized by increases in V_{max} values with no changes in the affinity for HCO_3^-. The increase in Cl^-/HCO_3^- exchanger activity in SHR PTE cells may reflect the overexpression of the Slc26a6 protein. It is likely that Slc26a6 may act as an effector protein for PKA, PKC and p38 MAPK.

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REFERENCES

[1] Mount DB, Romero MF. The SLC26 gene family of multifunctional anion exchangers. Pflugers Arch 2004;447:710–21.
[2] Knauf F, Yang CL, Thomson RB, Mentone SA, Giebisch G, Aronson PS. Identification of a chloride–formate exchanger expressed on the brush border membrane of renal proximal tubule cells. Proc Natl Acad Sci USA 2001;98: 9425–30.
[3] Petrovic S, Ma L, Wang Z, Soleimani M. Identification of an apical Cl^-/HCO_3 exchanger in rat kidney proximal tubule. Am J Physiol Cell Physiol 2003;285:C608–17.
[4] Alpern RJ. Apical membrane chloride/base exchange in the rat proximal convoluted tubule. J Clin Invest 1987;79: 1026–30.
[5] Aronson PS, Giebisch G. Mechanisms of chloride transport in the proximal tubule. Am J Physiol 1997;273:F179–92.
[6] Pedrosa R, Jose PA, Soares-da-Silva P. Defective D1-like receptor-mediated inhibition of the Cl^-/HCO_3^- exchanger in immortalized SHR proximal tubular epithelial cells. Am J Physiol Renal Physiol 2004;286:F1120–6.
[7] Albrecht FE, Drago J, Felder RA, Printz MP, Eisner GM, Robillard JE, et al. Role of the D_{1A} dopamine receptor in the pathogenesis of genetic hypertension. J Clin Invest 1996;97:2283–8.
[8] Hussain T, Lokhandwala MF. Altered arachidonic acid metabolism contributes to the failure of dopamine to inhibit Na^+, K^+-ATPase in kidney of spontaneously hypertensive rats. Clin Exp Hypertens 1996;18: 963–74.
[9] Pedrosa R, Gomes P, Hopfer U, Jose PA, Soares-da-Silva P. Gialpha3 protein-coupled dopamine D3 receptor-mediated inhibition of renal NHE3 activity in SHR proximal tubular cells is a PLC–PKC-mediated event. Am J Physiol Renal Physiol 2004;287:F1059–66.
[10] Pedrosa R, Gomes P, Soares-da-Silva P. Distinct signalling cascades downstream to Gsalpha coupled dopamine D1-like NHE3 inhibition in rat and opossum renal epithelial cells. Cell Physiol Biochem 2004;14:91–100.
[11] Pedrosa R, Gomes P, Zeng C, Hopfer U, Jose PA, Soares-da-Silva P. Dopamine D3 receptor-mediated inhibition of Na^+/H^+ exchanger activity in normotensive and spontaneously hypertensive rat proximal tubular epithelial cells. Br J Pharmacol 2004;142:1343–53.

[12] Xu J, Li XX, Albrecht FE, Hopfer U, Carey RM, Jose PA. Dopamine$_1$ receptor, G_{salpha}, and Na^+–H^+ exchanger interactions in the kidney in hypertension. Hypertension 2000;36:395–9.
[13] Alvarez BV, Vilas GL, Casey JR. Metabolon disruption: a mechanism that regulates bicarbonate transport. EMBO J 2005;24:2499–511.
[14] Hassan HA, Mentone S, Karniski LP, Rajendran VM, Aronson PS. Regulation of anion exchanger Slc26a6 by protein kinase C. Am J Physiol Cell Physiol 2007;292: C1485–92.
[15] Pedrosa R, Simão S, Fraga S, Soares-da-Silva P. Role of protein kinase C on the regulation of enhanced Cl^-/HCO_3^- exchanger (SLC26A6) activity in hypertension. Nephrol Dial Transplant 2006;21(Suppl. 4):iv307.
[16] Pedrosa R, Soares-da-Silva P. AT1 receptors mediate the enhanced sensitivity to angiotensin II and upon overexpressed Cl^-/HCO_3^- exchanger (SLC26A6) in hypertension. Nephrol Dial Transplant 2006;21(Suppl. 4):iv24.
[17] Simão S, Fraga S, Pedrosa R, Soares-da-Silva P. Norepinephrine and Cl^-/HCO_3^- exchanger activity in immortalized SHR proximal tubular epithelial cells. Nephrol Dial Transplant 2006;21(Suppl. 4):iv23–4.
[18] Woost PG, Orosz DE, Jin W, Frisa PS, Jacobberger JW, Douglas JG, et al. Immortalization and characterization of proximal tubule cells derived from kidneys of spontaneously hypertensive and normotensive rats. Kidney Int 1996;50:125–34.
[19] Thomas JA, Buchsbaum RN, Zimniak A, Racker E. Intracellular pH measurements in Ehrlich ascites tumor cells utilizing spectroscopic probes generated in situ. Biochemistry 1979;18:2210–8.
[20] Pedrosa R, Goncalves N, Hopfer U, Jose PA, Soares-da-Silva P. Activity and regulation of Na^+–HCO_3^- cotransporter in immortalized spontaneously hypertensive rat and Wistar–Kyoto rat proximal tubular epithelial cells. Hypertension 2007;49:1186–93.
[21] Magro F, Fraga S, Soares-da-Silva P. Signaling of short- and long-term regulation of intestinal epithelial type 1 Na^+/H^+ exchanger by interferon-gamma. Br J Pharmacol 2005;145:93–103.
[22] Gomes P, Vieira-Coelho MA, Soares-da-Silva P. Ouabain-insensitive acidification by dopamine in renal OK cells: primary control of the Na(+)/H(+) exchanger. Am J Physiol Regul Integr Comp Physiol 2001;281:R10–8.
[23] Fraga S, Luo Y, Jose P, Zandi-Nejad K, Mount DB, Soares-da-Silva P. Dopamine D1-like receptor-mediated inhibition of Cl/HCO_3^- exchanger activity in rat intestinal epithelial IEC-6 cells is regulated by G protein-coupled receptor kinase 6 (GRK 6). Cell Physiol Biochem 2006;18:347–60.
[24] Gomes P, Soares-da-Silva P. Role of cAMP-PKA–PLC signaling cascade on dopamine-induced PKC-mediated inhibition of renal Na^+–K^+-ATPase activity. Am J Physiol Renal Physiol 2002;282:F1084–96.
[25] Gomes P, Soares-da-Silva P. Dopamine acutely decreases type 3 Na^+/H^+ exchanger activity in renal OK cells through the activation of protein kinases A and C signalling cascades. Eur J Pharmacol 2004;488:51–9.
[26] Alexandrov A, Keffel S, Goepel M, Michel MC. Stimulation of alpha1A-adrenoceptors in Rat-1 cells inhibits extracellular signal-regulated kinase by activating p38 mitogen-activated protein kinase. Mol Pharmacol 1998;54:755–60.
[27] Markou T, Lazou A. Phosphorylation and activation of mitogen- and stress-activated protein kinase-1 in adult rat cardiac myocytes by G-protein-coupled receptor agonists requires both extracellular-signal-regulated kinase and p38 mitogen-activated protein kinase. Biochem J 2002;365: 757–63.
[28] Snabaitis AK, Yokoyama H, Avkiran M. Roles of mitogen-activated protein kinases and protein kinase C in alpha$_{1A}$-adrenoceptor-mediated stimulation of the sarcolemmal Na^+–H^+ exchanger. Circ Res 2000;86:214–20.
[29] Aronson PS. Ion exchangers mediating Na^+, HCO_3^- and Cl^- transport in the renal proximal tubule. J Nephrol 2006;19(Suppl. 9):S3–10.
[30] Soleimani M. Expression, regulation and the role of SLC26 Cl^-/HCO_3^- exchangers in kidney and gastrointestinal tract. Novartis Found Symp 2006;273:91–102. discussion 103–6, 261–4.
[31] Soodvilai S, Chatsudthipong V, Evans KK, Wright SH, Dantzler WH. Acute regulation of OAT3-mediated estrone sulfate transport in isolated rabbit renal proximal tubules. Am J Physiol Renal Physiol 2004;287:F1021–9.
[32] Soodvilai S, Wright SH, Dantzler WH, Chatsudthipong V. Involvement of tyrosine kinase and PI3K in the regulation of OAT3-mediated estrone sulfate transport in isolated rabbit renal proximal tubules. Am J Physiol Renal Physiol 2005;289:F1057–64.
[33] Hanson AS, Linas SL. Parathyroid hormone/adenylate cyclase coupling in vascular smooth muscle cells. Hypertension 1994;23:468–75.
[34] Woodcock EA, Johnston CI. Selective inhibition by epinephrine of parathyroid hormone-stimulated adenylate cyclase in rat renal cortex. Am J Physiol 1982;242:F721–6.
[35] Magro F, Fraga S, Ribeiro T, Soares-da-Silva P. Intestinal Na^+–K^+-ATPase activity and molecular events downstream of interferon-gamma receptor stimulation. Br J Pharmacol 2004;142:1281–92.
[36] Magro F, Fraga S, Soares-da-Silva P. Interferon-gamma-induced STAT1-mediated membrane retention of NHE1 and associated proteins ezrin, radixin and moesin in HT-29 cells. Biochem Pharmacol 2005;70:1312–9.