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Dopamine D_3 receptor-mediated inhibition of Na^+/H^+ exchanger activity in normotensive and spontaneously hypertensive rat proximal tubular epithelial cells

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1 This study evaluated the response of the Na^+/H^+ exchanger (NHE) to dopamine D_1- and D_2-like receptor stimulation in immortalized renal proximal tubular epithelial cells and freshly isolated renal proximal tubules from the spontaneously hypertensive rat (SHR) and their normotensive controls (Wistar Kyoto rats; WKY).

2 Stimulation of D_1-like receptors with SKF 38393 attenuated NHE activity in WKY cells ($IC_{50} = 151$ nM), but not in SHR cells. Stimulation of D_2-like receptors with quinerolane ($IC_{50} = 120$ nM) attenuated NHE activity in SHR cells, but not in WKY cells. Forskolin was equipotent in SHR and WKY cells in inhibiting NHE activity. The effect of SKF 38393 was abolished by overnight treatment of WKY cells with cholera toxin (CTX, 500 ng ml^{-1}), but not with pertussis toxin (PTX, 100 ng ml^{-1}). The effect of quinerolane (1 μM) was abolished by overnight treatment of SHR cells with PTX, but not with CTX.

3 The D_3 receptor agonist 7-OH-DPAT ($IC_{50} = 0.8$ μM) attenuated NHE activity in SHR cells only. This effect was abolished by *S*-sulpiride and by overnight treatment with PTX. The D_4 receptor agonist RBI 257 did not affect NHE activity.

4 The 7-OH-DPAT inhibited NHE activity in freshly isolated renal proximal tubules from 4- and 12-week-old SHR and 12-week-old WKY, but not in freshly isolated renal proximal tubules from 4-week-old WKY.

5 It is concluded that D_3 receptors coupled to a $G_{i/o}$ protein play a role in the handling of tubular Na^+, namely through inhibition of the NHE activity, this being of particular relevance in the SHR, which fail to respond to D_1-like dopamine receptor stimulation.
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Abbreviations: BCECF-AM, 2′,7′-*bis*(carboxyethyl)-5(6)-carboxyfluorescein; CTX, cholera toxin; EIPA, ethylisopropylamiloride; NHE, Na^+/H^+ exchanger; PTX, pertussis toxin; SHR, spontaneously hypertensive rat; WKY, Wistar Kyoto rat

Introduction

A considerable part of the transepithelial absorption of sodium occurs across the renal proximal tubule through the involvement of two key proteins: the apical Na^+/H^+ exchanger (NHE) and the basolateral Na^+–K^+–ATPase. These two membrane transporters have been identified as targets for the action of dopamine (Aperia *et al*., 1987; Felder *et al*., 1990; Jose *et al*., 1992).

Dopamine produced by renal proximal tubular cells exerts an autocrine/paracrine action *via* two classes of dopamine receptors, D_1 like (D_1 and D_5) and D_2 like (D_2, D_3, and D_4), that are differentially expressed along the nephron (Felder *et al*., 1984; Lokhandwala & Amenta, 1991). The autocrine/paracrine function of dopamine, manifested by tubular rather than by hemodynamic mechanisms, becomes most evident during extracellular fluid volume expansion (Jose *et al*., 1998). This renal autocrine/paracrine function is lost in essential hypertension and in some animal models of genetic hypertension (Aoki *et al*., 1989; Kinoshita *et al*., 1989; Iimura & Shimamoto, 1990; Yoshimura *et al*., 1990; Horiuchi *et al*., 1992; Hussain & Lokhandwala, 1997). Furthermore, disruption of the D_1 or D_3 receptor produces hypertension in mice (Albrecht *et al*., 1996; Asico *et al*., 1998). In some humans with essential hypertension, renal dopamine production in response to sodium loading is often impaired and may contribute to the hypertension (Shikuma *et al*., 1986). However, urinary dopamine is higher in young adults with hypertension than normotensive controls, indicating abnormality at the receptor or postreceptor levels (Saito *et al*., 1994). The molecular basis for the dopaminergic dysfunction in hypertension is not known, but may involve an abnormal post-translational modification of the dopamine receptor. There may be a

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primary defect in D_1-like receptors and an altered signaling system in the proximal tubules that lead to reduced dopamine-mediated effects on renal sodium excretion in hypertension (Jose *et al*., 1998).

In the spontaneously hypertensive rat (SHR), in spite of normal renal production of dopamine and receptor density, there is defective transduction of the D_1 receptor signal in renal proximal tubules. This coupling defect is genetic (precedes the onset of hypertension and cosegregates with the hypertensive phenotype), is receptor specific (not shared by other humoral agents) and is organ and nephron segment selective (occurs in proximal tubules, but not in cortical collecting ducts or the brain striatum) (Kinoshita *et al*., 1989; Felder *et al*., 1993; Ohbu & Felder, 1993; Jose *et al*., 1998). A consequence of the defective dopamine receptor/adenylyl cyclase coupling in the SHR is a decreased ability of D_1-like receptor agonists to inhibit Na^+/H^+ exchange activity (Horiuchi *et al*., 1992; Albrecht *et al*., 1996; Jose *et al*., 1998; Xu *et al*., 2000). A resistance to the natriuretic effect of dopamine and D_1-like receptor agonists in the SHR is due mainly to decreased cyclic AMP production, although with maturation a postcyclic AMP defect is acquired (Horiuchi *et al*., 1992). In contrast with the considerable amount of information on D_1-like receptors in hypertension, there is scarce evidence on the role of renal D_2-like receptors in hypertension. Although some studies have described abnormalities of D_2-like receptors in genetic hypertension (Vachvanichsanong *et al*., 1996; Ladines *et al*., 2001), a systematic evaluation on the role of renal tubular D_2-like receptors has not been performed in hypertension. However, it has been reported that in hypertension the interaction between D_1- and D_2-like receptors to regulate renal vasodilatation, diuresis and natriuresis may be impaired (Vachvanichsanong *et al*., 1996; Ladines *et al*., 2001), whereas others suggested a diminished expression and function of dopamine D_3 receptors in genetically salt-sensitive Dahl rats (Luippold *et al*., 2001).

The present study was carried out to evaluate the response of NHE to D_1- and D_2-like receptor stimulation in immortalized renal proximal tubular epithelial cells and freshly isolated renal proximal tubules from SHR and their normotensive controls (Wistar Kyoto rats; WKY).

Methods

Cell culture

Immortalized renal proximal tubular epithelial cells from 4- to 8-week-old WKY and SHR animals (Woost *et al*., 1996) were maintained in a humidified atmosphere of 5% CO_2–95% air at 37°C. SHR and WKY cells (SHR, passages 77–123; WKY, passages 42–110) were grown in Dulbecco's modified Eagle's medium nutrient mixture F-12 Ham (Sigma Chemical Company, St Louis, MO, U.S.A.) supplemented with 100 U ml^{-1} penicillin G, 0.25 μg ml^{-1} amphotericin B, 100 μg ml^{-1} streptomycin (Sigma), 4 μg ml^{-1} dexamethasone (Sigma), 5 μg ml^{-1} transferrin (Sigma), 5 μg ml^{-1} insulin (Sigma), 5 ng ml^{-1} selenium (Sigma), 10 ng ml^{-1} 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 : 4 and subcultured in Costar plates with 21-cm^2 growth areas (Costar, Badhoevedorp, The Netherlands). For intracellular pH measurement experiments, cells were grown in 96-well plates (Costar). The cell medium was changed every 2 days, and the cells reached confluence after 3–5 days of incubation. For 24 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.

Preparation of renal proximal tubules

SHR and WKY rats (Harlan, Barcelona, Spain) 4 and 12 weeks old and weighing 60–80 and 310–340 g, respectively, were used in the experiments. Animals were kept two per cage under controlled environmental conditions (12 h light/dark cycle and room temperature 24°C). Food and tap water were allowed *ad libitum* and the experiments were all carried out during daylight hours. The preparation of renal tubules was based on the techniques previously described and was found to contain predominantly proximal tubules (Soares-da-Silva *et al*., 1994; 1995). In brief, after anesthesia (sodium pentobarbital, 60 mg kg^{-1}, i.p.), a midline incision was performed, the aorta was cannulated with polyethylene catheters and both kidneys were perfused with Hanks' solution (in mM): 137 NaCl, 5 KCl, 0.8 $MgSO_4$, 0.33 Na_2PO_4, 0.44 KH_2PO_4, 0.25 $CaCl_2$, 1.0 $MgCl_2$, 15 Tris-HCl and 1.0 sodium butyrate, pH = 7.4. Thereafter, the kidneys were removed through a midline abdominal incision, after which they were decapsulated, cut in half and placed in ice-cold Hanks' solution. The outer cortex was cut out with fine scissors and minced with a scalpel into a fine paste. The cortical paste was filtered sequentially through a series of Nybolt nylon sieves, first 180 μm and then 75 μm. Undissociated cortex remained on the upper (180 μm) sieve, while the lower one (75 μm) retained predominantly proximal nephron segments. The sieves were continuously rinsed with cold Hanks' solution. The retained tubules were then washed with cold Hanks' solution and collected into a pellet by centrifugation at 200 × *g*, 5 min, 4°C; renal tubules used in incubation experiments were suspended in Hanks' solution. The viability of proximal renal tubules used in this study was assessed by the trypan blue (0.2% (w v^{-1})) exclusion method; nephron segments were pipetted to a glass slide and observed 90 s after exposure to the dye, using a Leica microscope. Under these conditions, more than 90% of the renal tubules excluded the dye.

Preparation of renal cortical membranes

The renal cortical membrane fractions were prepared in TE buffer (mM, 10 Tris-Cl, pH 7.4, 2 EDTA) and TBST buffer (mM, 10 Tris-Cl, pH 7.4, 150 NaCl, 2 EDTA, 0.5% Triton X-100) as described previously with modification (Yu *et al*., 1995). All buffers contained protease inhibitors (mM): 5 DTT, 2 EDTA, and 1 pofabloc and 10 μg ml^{-1} each of leupeptin and aprotinin.

NHE activity

NHE activity was assayed as the initial rate of pH_i recovery after an acid load imposed by 20 mM NH_4Cl followed by the removal of Na^+ from the Krebs' modified buffer solution (in

mM: NaCl 140, KCl 5.4, $CaCl_2$ 2.8, $MgSO_4$ 1.2, NaH_2PO_4 0.3, KH_2PO_4 0.3, HEPES 10, glucose 5, pH = 7.4, adjusted with Tris base), in the absence of CO_2/HCO_3 (Hoinard & Gore, 1988; Gore & Hoinard, 1989). In these experiments, NaCl was replaced by an equimolar concentration of tetramethylammonium chloride (TMA). Test compounds were added to the extracellular fluid during the acidification and Na^+-dependent pH_i recovery periods. The concentration response relationship of the initial rate of pH_i recovery for extracellular Na^+ was evaluated by bathing the apical side of the monolayers with a modified Krebs–Hensleit solution over a range of Na^+ concentrations from 0 to 143 mM (NaCl replaced with TMA) without affecting the concentrations of other ions.

Intracellular pH was measured, as described previously (Gomes *et al*., 2001). The renal proximal tubules were incubated at 37°C for 30 min with 5 μM of the acetoxymethyl ester of 2′,7′-*bis*(carboxyethyl)-5(6)-carboxyfluorescein (BCECF-AM). Renal proximal tubules were then washed (centrifugation at $100 \times g$) twice with prewarmed dye-free modified Krebs' buffer before initiation of the fluorescence recordings. Test compounds were added to the incubation medium 25 min before starting the sodium-dependent pH_i recovery period. Renal proximal tubules were then placed in a 1×1-cm^2 acrylic fluorometric cuvettes inserted in a Perkin-Elmer cuvette holder (model LS 50), and subsequently placed in the sample compartment of a FluoroMax-2 spectrofluorometer (Jobin Yvon-SPEX, Edison, NJ, U.S.A.). The cuvette volume of 2.0 ml was constantly stirred and maintained at 37°C. After 5 min, fluorescence was measured every 5 s alternating between 440 and 490 nm excitation (1 nm slit size) at 525 nm emission (3 nm slit size) and 5 min later 100 μl of 2.8 M NaCl were injected into the cuvette to initiate the sodium-dependent pH_i recovery period. In some experiments, intracellular pH measurements were performed in SHR and WKY cells cultured in 96-well plates (Gomes *et al*., 2001). After loading the cells with 5 μM BCECF-AM at 37°C for 30 min, test compounds were added to the extracellular fluid 25 min before starting the sodium-dependent pH_i recovery period. Cells were placed in the sample compartment of a dual-scanning microplate spectrofluorometer (Spectramax Gemini, Molecular Devices, Sunnyvale, CA, U.S.A.), and fluorescence was measured every 19 s alternating between 440 and 490 nm excitation at 535 nm emission, with a cutoff filter of 530 nm. In both types of experiments, the ratio of intracellular BCECF fluorescence at 490 and 440 nm was converted to intracellular pH values by comparison with values from an intracellular calibration (performed for each day of experiment) curve using the nigericin (10 μM) and high-K^+ method (Thomas *et al*., 1979).

Immunoblotting studies

The immortalized renal proximal tubular epithelial cells and freshly renal cortical membranes from SHR and WKY were washed with phosphate-buffered saline (PBS) two to three times, lysed by brief sonication in PBS and centrifuged at 14,000 r.p.m. in an Eppendorf desk top refrigerated centrifuge. The pellets were resuspended with ice-cold lysis buffer (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% NP-40, 1 mM PMSF, and 10 $\mu g\,ml^{-1}$ each leupeptin and aprotinin), sonicated briefly and incubated on ice for 1 h. After centrifugation (Eppendorf table top centrifuge, 14,000 r.p.m. $\times$ 30 min), the supernatant was mixed in $6 \times$ sample buffer (0.5 M Tris-HCl, pH 6.8, 7 ml, glycerol 3 ml, SDS 1.04 g, DTT 0.93 g, bromphenol blue 1.2 mg, q.s. H_2O to 10 ml) and boiled for 5 min. The proteins were subjected to SDS–PAGE (8% SDS–polyacrylamide gel) and electrophoretically transferred onto nitrocellulose membranes. The transblot sheets were blocked with 5–10% nonfat dry milk in 25 mM Tris-HCl, pH 7.5, 150 mM NaCl and 0.1% Tween-20 overnight at 4°C. Then, the membranes were incubated with appropriately diluted antibodies or antisera and the reaction detected by peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.) and ECL system (Amersham Life, Arlington Heights, IL, U.S.A.). Specificity of the affinity-purified NHE3 antibody was determined by the use of preimmune sera or antibody preadsorbed with immunizing peptide, as described previously (Xu *et al*., 2000). Polyclonal antibodies to the NHE1 were obtained from Chemicon International Inc. (Tempecula, CA, U.S.A.). The densities of the appropriate bands were determined using Quantiscan (Biosoft, Ferguson, MO, U.S.A.). Protein concentration was measured using bovine serum albumin as standard.

Cyclic AMP measurement

Cyclic AMP was determined with an enzyme immunoassay kit (Amersham Pharmacia Biotech, Little Chalfont, U.K.), as described previously (Cheng *et al*., 1990). Cells were pre-incubated for 15 min at 37°C in Hanks' medium (in mM: NaCl 137, KCl 5, $MgSO_4$ 0.8, Na_2HPO_4 0.33, KH_2PO_4 0.44, $CaCl_2$ 0.25, $MgCl_2$ 1.0, Tris-HCl 0.15 and sodium butyrate 1.0, pH = 7.4), containing 100 μM 3-isobutyl-1-methylxanthine, a phosphodiesterase inhibitor. Cells were then incubated for 25 min with forskolin, SKF 38393 (D_1-like agonist) or quinerolane (D_2-like agonist). At the end of the experiment, cells were lysed by the addition of 200 μl lysis reagent. Aliquots were taken for the measurement of total cyclic AMP content.

Data analysis

The K_m and V_{max} values for Na^+ dependence of NHE activity were calculated from nonlinear regression analysis using the GraphPad Prism statistics software with the equation: $Y = V_{max}X/(K_m + X)$, where Y represents the NHE activity (dpH/dt, pH units/s) and X the Na^+ concentration. The IC_{50} was calculated from nonlinear regression analysis using the GraphPad Prism statistics software with the equation: $Y = \text{bottom} + (\text{top–bottom})/(1 + 10^{(X - \text{LogIC}_{50})})$; Y represents the NHE activity and X the Log[concentration]. Arithmetic means are given with s.e.m. or geometric means with 95% confidence values. Statistical analysis was carried out with a one-way analysis of variance (ANOVA) followed by Newman–Keuls test for multiple comparisons. A P-value less than 0.05 was assumed to denote a significant difference.

Drugs

Amiloride, forskolin, (±)-SKF 83566 hydrochloride, *S*(−)-sulpiride, (±)-SKF-38393 hydrochloride, *R*-(+)-7-hydroxy-DPAT, RBI 257 maleate and quinerolane hydrochloride were purchased from Sigma Chemical Company, St Louis, MO, U.S.A. Acetoxymethyl ester of BCECF-AM,

ethylisopropylamiloride (EIPA) and nigericin were obtained from Molecular Probes (Eugene, OR, U.S.A.).

Results

NHE activity in WKY and SHR immortalized cells

NHE activity was assayed as the initial rate of pH_i recovery measured after an acid load imposed by 20 mM NH_4Cl followed by the removal of Na^+ from the Krebs' modified buffer solution, in the absence of CO_2/HCO_3 (Figure 1a). As shown in the figure, the Na^+-dependent recovery of pH_i in SHR cells was steeper than that observed in WKY cells. The pH_i recovery rates (in dpH_i/dt, pH units/s) during the linear phase of pH_i recovery after intracellular acidification in SHR cells was greater than that in WKY cells (Figure 1b). To define whether the steeper Na^+-dependent recovery of pH_i in SHR cells was related to increases in maximal activity of the transporter or enhanced affinity for Na^+ or H^+, pH_i recovery was evaluated at increasing extracellular concentrations of Na^+ (0–140 mM) and increasing extracellular pH (5.8–7.4), respectively. As shown in Figure 2a, the recovery of pH_i was clearly a Na^+-dependent process in both SHR and WKY cells. However, the maximal rate at which the pH_i recovery occurred

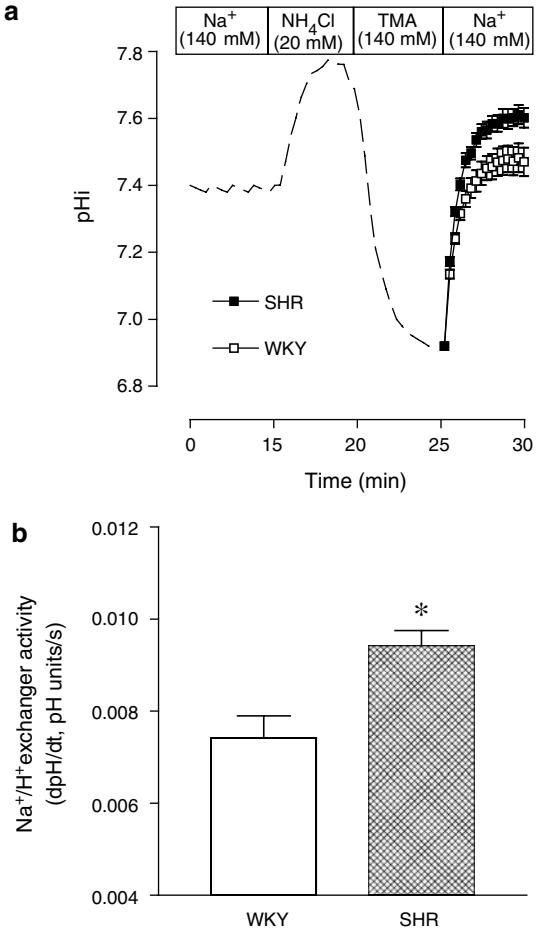

Figure 1 Assessment of (a) intracellular pH and (b) NHE activity under V_{max} conditions as the initial rate of Na^+-dependent pH_i recovery after an acid load imposed by exposure to NH_4Cl followed by Na^+ removal of the perfusion medium in WKY and SHR cells. Traces represent means of nine to 14 experiments per group. Columns represent the mean of nine to 15 independent determinations; vertical lines show s.e.m. Significantly different from values in WKY cells ($*P<0.05$).

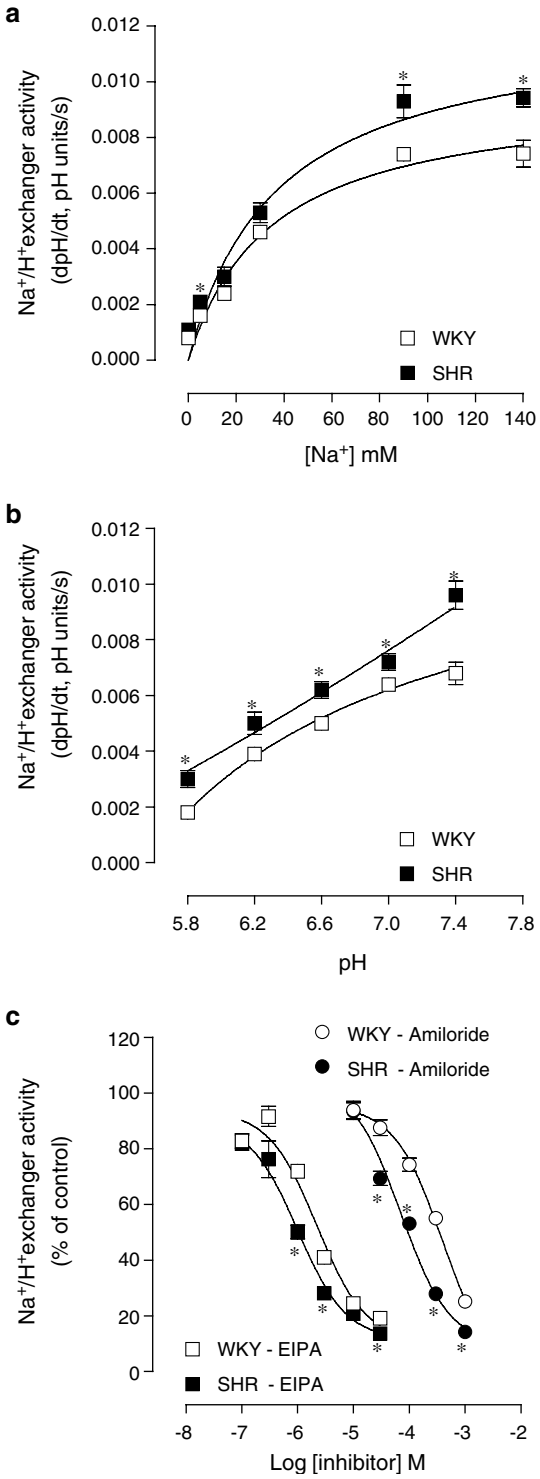

Figure 2 Influence of (a) extracellular Na^+, (b) extracellular pH and (c) amiloride (10–1000 μM) and EIPA(0.1–30 μM) on NHE activity in WKY and SHR cells. Symbols represent the mean of six to 15 independent determinations; vertical lines show s.e.m. Significantly different from values in WKY cells ($*P<0.05$).

in SHR cells was greater than that in WKY cells. This is also evidenced by the fact that V_{max} values (in pH units/s) for Na^+-dependent pH_i recovery in SHR cells (0.0144 ± 0.0011) were greater ($P<0.05$) than those in WKY (0.0097 ± 0.0007), with similar K_m values (in mM) for Na^+ (WKY, 35 ± 9; SHR, 50 ± 13). The view that Na^+ removal reduced approximately 90% of the recovery of pH_i excludes the contribution of the H-ATPase and clearly demonstrated a specific contribution of NHE activity during the alkalinization process after the Na^+ removal. The recovery of pH_i was also clearly dependent on extracellular pH in both SHR and WKY cells. However, at all extracellular pH values, but most evident at pH = 7.4, the Na^+-dependent pH_i recovery in SHR cells was greater than that in WKY cells (Figure 2b). The sensitivity of the NHE to inhibition by amiloride and EIPA was also evaluated. As indicated in Figure 2c, both amiloride and EIPA produced marked inhibition of NHE activity in SHR and WKY cells, EIPA being considerably more potent than amiloride. Both compounds EIPA and amiloride were significantly more potent in inhibiting NHE activity in SHR cells than in WKY cells. Differences in sensitivity to amiloride and EIPA are in agreement with the observation that rat renal proximal tubules express mainly the type 3 NHE (NHE3) (Biemesderfer *et al*., 1997; Karim *et al*., 1999).

The addition of dopamine (1 μM) during the acidification and recovery periods markedly attenuated the sodium-dependent recovery of pH_i in both WKY cells and SHR cells (Table 1). The inhibitory effect of dopamine in WKY cells was antagonized by the selective D_1-like receptor antagonist SKF 83566 (1 μM) and insensitive to the selective D_2-like receptor antagonist *S*-sulpiride (1 μM) (Table 1). In contrast, the inhibitory effect of dopamine in SHR cells was antagonized by the selective D_2-like receptor antagonist *S*-sulpiride (1 μM) and insensitive to the selective D_1-like receptor antagonist SKF 83566 (1 μM) (Table 1). Stimulation of D_1-like receptors with SKF 38393 (1 μM) during the acidification and recovery periods markedly attenuated the sodium-dependent recovery of pH_i in WKY cells, but not in SHR cells (Table 1). In contrast, stimulation of D_2-like receptors with quinerolane (1 μM) during the acidification and recovery periods markedly attenuated the sodium-dependent recovery of pH_i in SHR cells, but not in WKY cells (Table 1).

Table 1 NHE activity (% of control) in WKY and SHR cells in the absence or presence of dopamine (1 μM), SKF 83566 (1 μM), *S*-sulpiride (1 μM), SKF 38393 (1 μM) and quinerolane (1 μM)

Treatment	*WKY*	*SHR*
Vehicle	100 ± 3	100 ± 3
Dopamine	82 ± 3*	83 ± 2*
S-sulpiride	100 ± 6	100 ± 4
S-sulpiride + dopamine	73 ± 4*, #	109 ± 6
SKF 83566	100 ± 4	100 ± 5
SKF 83566 + dopamine	106 ± 7	80 ± 5*, #
S-sulpiride + SKF 83566	100 ± 5	100 ± 4
S-sulpiride + SKF 83566 + dopamine	97 ± 4	112 ± 5
SKF 38393	70 ± 3*	91 ± 4
Quinerolane	91 ± 8	74 ± 3*

Values are means $\pm$ s.e.m. Significantly different from values for vehicle (*$P<0.05$) and corresponding control values (#$P<0.05$).

As resistance to the natriuretic effect of dopamine and D_1-like receptor agonists in the SHR is due mainly to decreased cyclic AMP production, although with maturation a postcyclic AMP defect is acquired (Horiuchi *et al*., 1992), it was felt worthwhile evaluating the sensitivity of the NHE in WKY and SHR cells to forskolin. As shown in Figure 3a, forskolin was equipotent in SHR cells ($IC_{50} = 9$ [4, 20] μM; means with 95% confidence limits) and WKY cells ($IC_{50} = 13$ [6, 28] μM) in inhibiting NHE activity. As shown in Figure 3b, the selective D_1-like receptor agonist SKF 38393 (10–10,000 nM) was found to attenuate, in a concentration-dependent manner ($IC_{50} = 151$ [13, 1761] nM), the Na^+-dependent pH_i recovery in WKY cells. Similarly, the selective D_2-like receptor agonist quinerolane (10–10,000 nM) attenuated, in a concentration-dependent manner ($IC_{50} = 120$ [6, 2402] nM), the Na^+-dependent pH_i recovery in SHR cells (Figure 3c). The effect of SKF 38393 (1 μM) on NHE activity in WKY cells (Table 2) and the effect of quinerolane (1 μM) in SHR cells (Table 3) was abolished by the selective receptor antagonists SKF 83566 (1 μM) and *S*-sulpiride (1 μM), respectively. Next, we evaluated the involvement of G proteins in the regulation of NHE activity in WKY and SHR cells. Cholera toxin (CTX) and pertussis toxin (PTX) ribosylate the α-subunit of the G_s and $G_{i/o}$ classes of G proteins, respectively. The effect of SKF 38393 (1 μM) was abolished by overnight treatment of WKY cells with CTX (500 ng ml^{-1}), but not with PTX (100 ng ml^{-1}) (Table 2). On the other hand, the effect of quinerolane (1 μM) was abolished by overnight treatment of SHR cells with PTX (100 ng ml^{-1}), but not with CTX (500 ng ml^{-1}) (Table 3). These results suggest that D_1-like receptors stimulated by SKF 38393 in WKY cells are coupled to CTX-sensitive G proteins of the G_s class, whereas D_2-like receptors stimulated by quinerolane in SHR cells are coupled to PTX-sensitive G proteins of the $G_{i/o}$ class.

Since D_2-like receptors include D_2, D_3 and D_4 receptors, and these have all been identified in the kidney, it was decided to dissect the D_2-like receptor-mediated responses using 7-OH-DPAT and RBI 257, that are relatively selective agonists of, respectively, D_3 (Freedman *et al*., 1994; Large & Stubbs, 1994) and D_4 receptors (Kula *et al*., 1997). As shown in Figure 4a, the D_3 receptor agonist 7-OH-DPAT (0.1–100 μM) attenuated, in a concentration-dependent manner ($IC_{50} = 0.8$ [0.1, 3.9] μM), the Na^+-dependent pH_i recovery in SHR cells, but not in WKY cells. The effect of 7-OH-DPAT (100 μM) on NHE activity in SHR cells was abolished by *S*-sulpiride (1 μM) and by overnight treatment of SHR cells with PTX (100 ng ml^{-1}) (Table 3). The D_4 receptor agonist RBI 257 (10–100 μM) did not affect the Na^+-dependent pH_i recovery in both SHR and WKY cells (Figure 4b).

Although the data presented here are in agreement with the observation that SHR and WKY cells express mainly NHE3, it is not apparent from the experiments shown above that D_1-like or D_3 receptor-mediated attenuation of the sodium-dependent recovery of pH_i in WKY and SHR cells is dependent on NHE3 inhibition. To evaluate whether these effects would involve inhibition of NHE3 or inhibition of NHE1, it was felt worthwhile to study the effects of SKF 38393 and 7-OH-DPAT in the presence of 10 μM amiloride. This concentration of amiloride is selective for the inhibition of NHE1 being devoid of effects upon NHE3 (Orlowski, 1993). The sodium-dependent recovery of pH_i in WKY cells was reduced by SKF 38393 (1 μM) to 68 ± 2 and 73 ± 1% of control in the presence

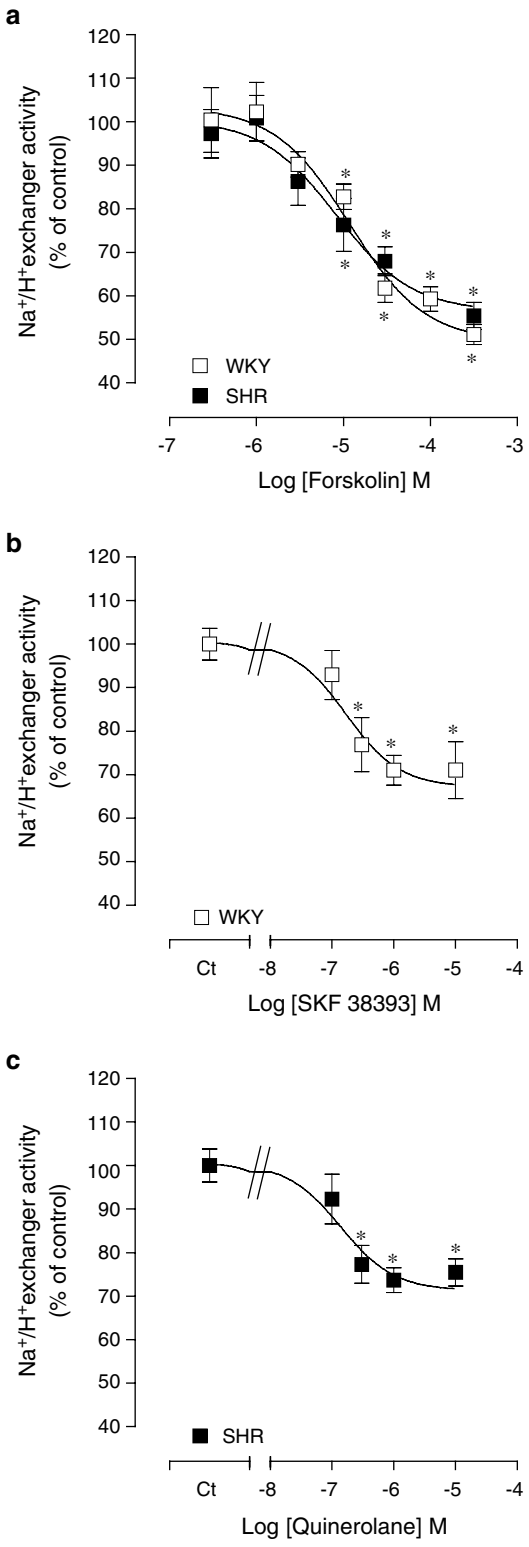

Figure 3 Concentration-dependent effect of (a) forskolin, (b) SKF 38393 and (c) quinerolane on NHE activity in WKY and SHR. Symbols represent the mean of seven to 32 experiments per group; vertical lines indicate s.e.m. Significantly different from corresponding control values ($*P<0.05$).

and absence of amiloride (10 μM), respectively. Similarly, there was no significant difference between reduction of the sodium-dependent recovery of pH_i in SHR cells by 7-OH-DPAT (100 μM) in the presence (83±4% of control) or absence (80±2% of control) of amiloride (10 μM), respectively. This strongly suggests that the NHE3 is the isoform involved in the response to both SKF 38393 and 7-OH-DPAT.

Table 2 NHE activity (% of control) in WKY and SHR cells in the absence and presence of SKF 38393 (1 μM), SKF 83566 (1 μM), CTX (500 ng ml^{-1}) or PTX (100 ng ml^{-1})

Treatment	*WKY*
Vehicle	100±4
SKF 38393	70±3*
SKF 83566	100±8
SKF 83566 + SKF 38393	102±4
CTX	100±9
CTX + SKF 38393	94±5
PTX	100±3
PTX + SKF 38393	78±3*, #

Values are means±s.e.m. Significantly different from values for vehicle ($*P<0.05$) and corresponding control values ($^{\#}P<0.05$).

Table 3 NHE activity (% of control) in SHR cells in the absence and presence of quinerolane (1 μM), 7-OH-DPAT (100 μM), *S*-sulpiride (1 μM), CTX (500 ng ml^{-1}) or PTX (100 ng ml^{-1})

Treatment	*SHR*
Vehicle	100±4
Quinerolane	74±3*
7-OH-DPAT	58±3*
S-sulpiride	100±5
S-sulpiride + quinerolane	94±4
S-sulpiride + 7-OH-DPAT	96±7
CTX	100±4
CTX + quinerolane	76±4*, #
CTX + 7-OH-DPAT	70±4*, #
PTX	100±8
PTX + quinerolane	106±6
PTX + 7-OH-DPAT	105±10

Values are means±s.e.m. Significantly different from values for vehicle ($*P<0.05$) and corresponding control values ($^{\#}P<0.05$).

NHE activity in WKY and SHR renal tubules

As observed in immortalized WKY and SHR cells, the maximal rate (in delta pH units/s) at which the pH_i recovery occurred in SHR renal tubules was greater than that in WKY renal tubules (Figure 5a). However, the difference in NHE activity between WKY and SHR renal tubules was more marked at 4 weeks than at 12 weeks (Figure 5a). Similarly to that observed in immortalized cells, amiloride produced marked inhibition of NHE activity in renal tubules from 12-week-old SHR and WKY. However, amiloride was almost equipotent in inhibiting NHE activity in WKY tubules and SHR tubules (Table 4). In the absence of extracellular Na^+ (NaCl was substituted by an equimolar concentration of choline chloride), the Na^+-dependent pH_i recovery was markedly attenuated in both 12-week-old WKY and SHR tubules (Table 4). In line with that obtained in immortalized cells, the removal of extracellular Na^+ reduced by 80% the

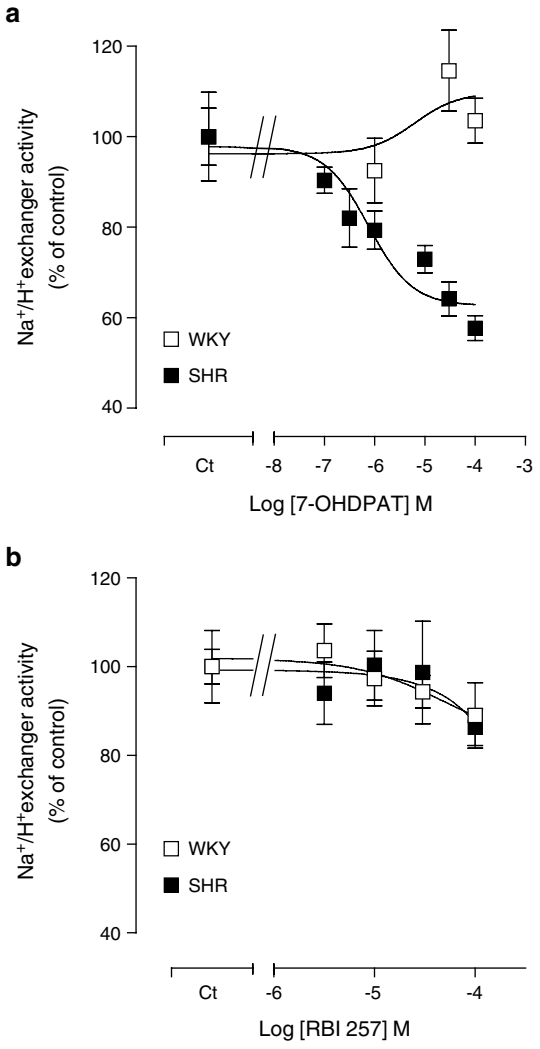

Figure 4 Concentration-dependent effect of (a) 7-OH-DPAT and (b) RBI 257 on NHE activity in WKY and SHR. Symbols represent the mean of five to 15 experiments per group; vertical lines indicate s.e.m.

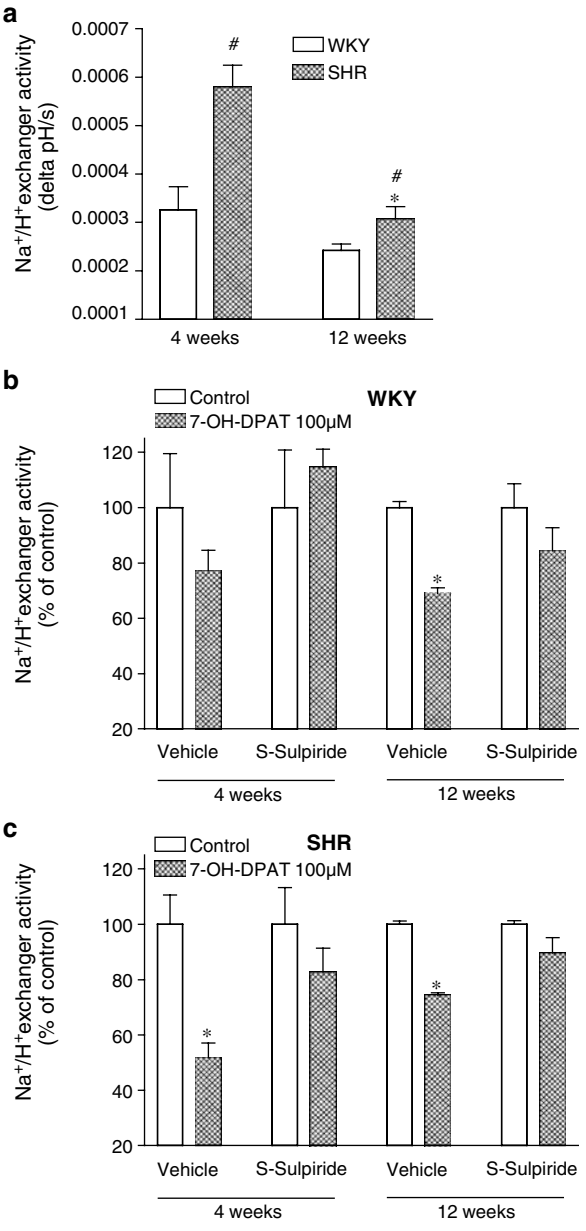

Figure 5 (a) NHE activity in isolated renal tubules from 4- and 12-week-old WKY and SHR. Changes in Na^+/H^+ activity in isolated renal tubules from (b) WKY and (c) SHR in control conditions and after exposure to 7-OH-DPAT (100 μM) and *S*-sulpiride (1 μM). Columns represent the mean of three to five experiments per group; vertical lines indicate s.e.m. Significantly different from corresponding values at 4 weeks and control values ($^*P<0.05$) or values for WKY ($^{\#}P<0.05$).

recovery of pH_i, which excludes the contribution of H^+-ATPase. As observed in immortalized cells, stimulation of D_3 receptors with 7-OH-DPAT (100 μM) during the acidification and recovery periods markedly attenuated the sodium-dependent recovery of pH_i in 4-week-old SHR, but not in 4-week-old WKY tubules (Figure 5b and c). In contrast, stimulation of D_3 receptors with 7-OH-DPAT (100 μM) during the acidification and recovery periods markedly attenuated the sodium-dependent recovery of pH_i in renal tubules from both 12-week-old WKY and SHR (Figure 5b and c). In the presence of the D_2-like receptor antagonist *S*-sulpiride (1 μM), 7-OH-DPAT (100 μM) failed to reduce NHE activity in WKY and SHR tubules (Figure 5b and c).

NHE expression

The expression of NHE1 and NHE3 was evaluated in immortalized renal proximal tubular epithelial cells and renal cortical membranes from 12-week-old WKY and SHR. As shown in Figure 6a, the level of expression of both NHE1 and NHE3 in SHR immortalized cells was greater than in WKY. The magnitude of the increase in NHE3 expression in SHR cells *versus* WKY cells was similar to that observed for NHE1 expression. The level of expression of NHE3 in renal cortical membranes from 12-week-old SHR was four-fold that in the WKY, which contrasted with that occurring with NHE1 expression. In fact, the expression of NHE1 in WKY renal cortical membranes was greater than SHR renal cortical membranes (Figure 6b).

Table 4 Changes in NHE activity (% of control) in WKY and SHR isolated renal tubules in control conditions and after exposure to amiloride (1 mM) and sodium-free medium

Treatment	*WKY*	*SHR*
Vehicle	100.0±5.2	100.0±8.2
Amiloride	62.7±4.4*	51.3±2.8*
Sodium free	22.7±9.0*	16.4±14.3*

Values are means±s.e.m. of five experiments per group. Significantly different from corresponding control values (*$P<0.05$).

Cyclic AMP measurements

As there were marked differences between SHR and WKY cells towards the sensitivity of NHE activity to inhibition by SKF 38393, but not to forskolin, it was decided to measure the accumulation of cyclic AMP in response to these agents. Both SHR and WKY cells responded to forskolin with increases in the formation of cyclic AMP (Figure 7a). However, only WKY responded to SKF 38393 with increases in the formation of cyclic AMP (Figure 7b), this increase being completely prevented by pretreatment with D_1-like receptor antagonist SKF 83566 (Figure 7c). Quinerolane did not change cyclic AMP levels in both WKY and SHR cells (Figure 7b).

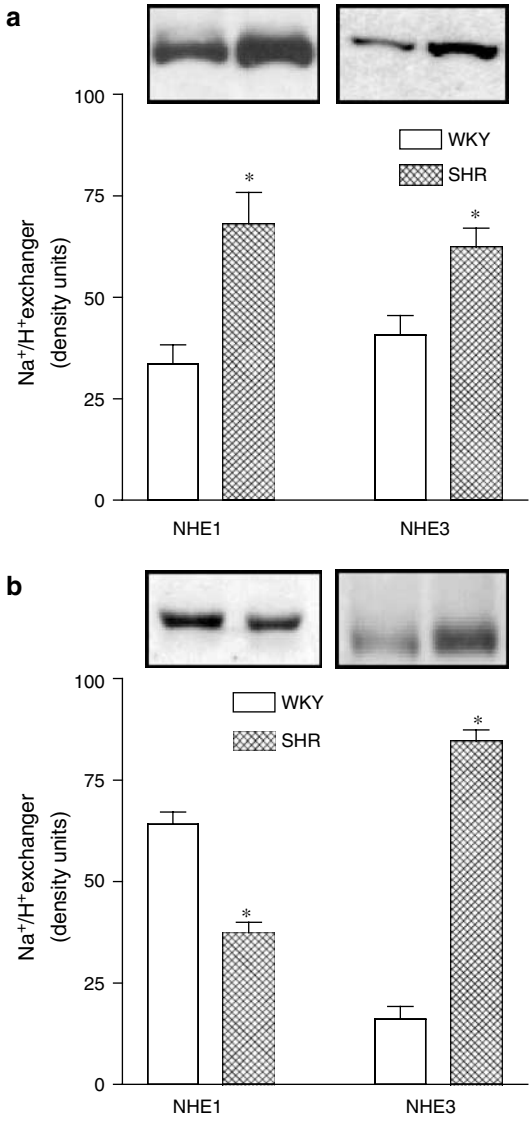

Figure 6 Expression of NHE1 and NHE3 in (a) immortalized renal proximal tubular epithelial cells and in (b) renal cortical membranes from WKY and SHR. Representative immunoblots are depicted on top of the bar graphs. Columns represent the mean of four to seven independent immunoblots; vertical lines show s.e.m. Significantly different from values in WKY cells (*$P<0.01$). NHE1 ~98 kDa, NHE3 ~85 kDa.

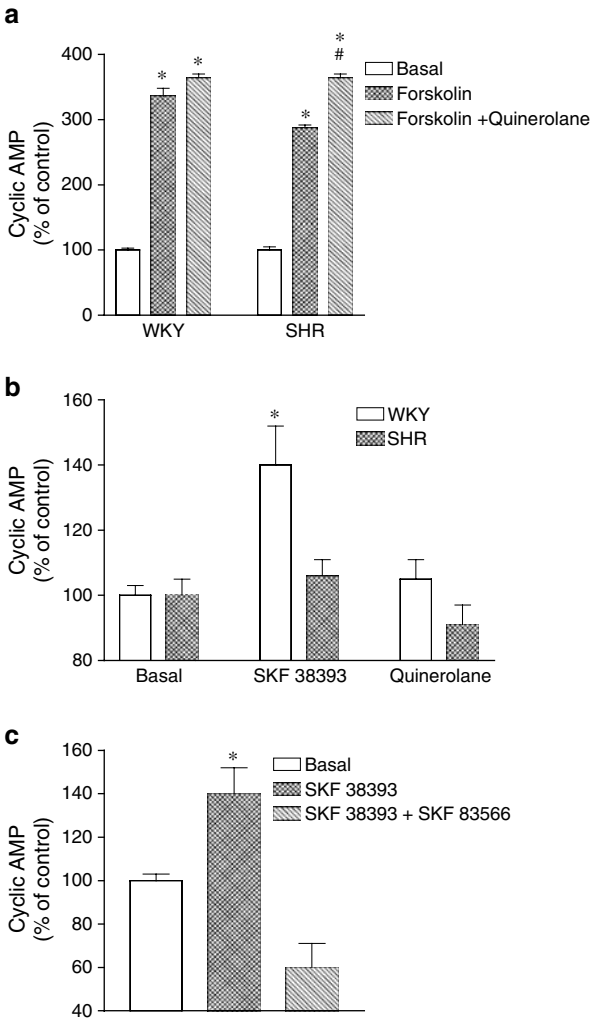

Figure 7 (a) Effect of forskolin (30 μM) on cyclic AMP accumulation in WKY and SHR cells in the absence and the presence of quinerolane (1 μM). (b) Effect of SKF 38393 (1 μM) and quinerolane (1 μM) on cyclic AMP accumulation in WKY and SHR cells. (c) Effect of SKF 38393 (1 μM) on cyclic AMP accumulation in WKY cells in the absence and the presence of SKF 83566 (1 μM). Each column is the mean of four or five separate experiments; vertical lines indicate s.e.m. Significantly different from corresponding control values (*$P<0.05$) or values for forskolin alone ($^{\#}P<0.05$).

Discussion

The present study was designed to evaluate the activity of the NHE in immortalized renal proximal tubular epithelial cells and freshly isolated renal proximal tubules from SHR and WKY and its sensitivity to inhibition during dopamine D_1- and D_2-like receptor stimulation. The results presented here confirm that freshly isolated and immortalized renal tubular cells from the SHR, but not their normotensive controls (WKY), have a defective transduction of the D_1 receptor signal downstream to the NHE. In addition, the present study shows that stimulation of dopamine D_3 receptors inhibits NHE activity in immortalized SHR, but not in WKY renal cells. The results obtained in freshly isolated renal tubules show that stimulation of dopamine D_3 receptors was able to inhibit NHE activity in renal tubules from 4- and 12-week-old SHR and 12-week-old WKY, but not in 4-week-old WKY. Considering that immortalized renal WKY and SHR cells were obtained from 4- to 8-week-old WKY and SHR animals, the present results suggest that differences in the ontogeny of dopamine D_3 receptors may occur between WKY and SHR animals. However, it is clear from the observations reported here that renal dopamine D_3 receptors play a role in the handling of tubular Na^+, namely through inhibition of the NHE activity. This is of particular relevance in the SHR where a defective D_1 receptor signal transduction has been well characterized before and during hypertension (Hussain & Lokhandwala, 1998; Jose *et al*., 1998; 2002; Li *et al*., 2001).

The results presented here show that dopamine significantly reduced the activity of NHE in both SHR and WKY cells. In WKY cells, the dopamine-induced inhibition of NHE activity was completely prevented by the selective D_1-like dopamine receptor antagonist SKF 83566, and insensitive to the selective D_2-like dopamine receptor antagonist *S*-sulpiride. In contrast, in SHR cells the inhibitory effect of dopamine was sensitive to *S*-sulpiride and insensitive to SKF 83566. These findings strongly suggest that dopamine-induced inhibition of the NHE in WKY cells is mediated through the activation of D_1-like dopamine receptors, whereas in SHR cells the effects of dopamine are mediated through D_2-like dopamine receptors. This pattern of NHE inhibition was confirmed with the use of selective agonist for D_1- and D_2-like dopamine receptors. The D_1-like receptor agonist SKF 38393 in WKY cells and the D_2-like receptor agonist quinerolane in SHR cells mimicked the effect of dopamine. On the other hand, the selective D_1-like receptor antagonist SKF 83566 and the selective D_2-like receptor antagonist *S*-sulpiride also antagonized the inhibitory effects of the selective agonists in WKY and SHR cells, respectively. The finding that in WKY cells the SKF 38393-induced inhibition of NHE activity was abolished by overnight treatment with CTX, but not with PTX, suggests that D_1-like receptors are coupled to a G_s class of G proteins. On the other hand, the finding that in SHR cells the quinerolane-induced inhibition of NHE activity was abolished by overnight treatment with PTX, but not with CTX, suggests that D_2-like receptors are coupled to a $G_{i/o}$ class of G proteins. However, this $G_{i/o}$ protein was not negatively coupled to adenylyl cyclase, as evidenced by the failure of quinerolane to reduce adenylyl cyclase activity. The defective dopamine receptor/adenylyl cyclase coupling in the SHR fits well the view that only WKY cells responded to SKF 38393 with increases in the formation of cyclic AMP and that forskolin in WKY and SHR cells was equipotent in inhibiting NHE activity, which paralleled with increases in the formation of cyclic AMP. The insensitivity of the renal NHE in the SHR to inhibition by D_1-like dopamine receptor agonists is in agreement with previous studies (Horiuchi *et al*., 1992; Albrecht *et al*., 1996; Xu *et al*., 2000).

To elucidate which type of D_2-like dopamine receptor was involved in the quinerolane-induced inhibition of NHE activity, it was decided to analyze the responses to 7-OH-DPAT and RBI 257, relatively selective agonists of, respectively, D_3 (Freedman *et al*., 1994; Large & Stubbs, 1994) and D_4 receptors (Kula *et al*., 1997). The D_3 receptor agonist 7-OH-DPAT decreased, in a concentration-dependent manner, NHE activity in SHR cells, but not in WKY cells. In contrast, the D_4 receptor agonist RBI 257 did not affect the Na^+-dependent pH_i recovery in both SHR and WKY cells. This finding is in agreement with previous reports showing that the most abundant D_2-like receptor in rat renal proximal tubules is the D_3 receptor (Jose *et al*., 1998; Luippold *et al*., 1998; O'Connell *et al*., 1998). The view that dopamine may induce natriuresis through dopamine D_3 receptor stimulation is in agreement with the observations of Luippold *et al*. (1998) showing that D_3 dopamine receptor activation increased the glomerular filtration rate (GFR), urinary volume and sodium excretion in Sprague–Dawley rats. However, in contrast to the present data, the same authors (Luippold *et al*., 2001) subsequently reported that the expression and the function of renal dopamine D_3 receptor were decreased in salt-sensitive Dahl rats, when compared with salt-resistant Dahl rats. This may be due to differences in the animal models used. However, it should be noted that the D_3 receptor density, as well as the dopamine D_3 receptor mRNA expression, was diminished in the kidneys of salt-sensitive Dahl when compared with salt-resistant Dahl rats (Muhlbauer *et al*., 2000; Luippold *et al*., 2001). Nevertheless, the increase in urinary sodium excretion-induced by 7-OH-DPAT was only impaired in salt-sensitive Dahl rats that were on a high-sodium diet. Recently, the renal response of adult SHR and WKY showing that D_3 dopamine receptor activation increased the GFR, urinary volume and sodium excretion (Luippold *et al*., 2003). We have also observed that the selective D_3 dopamine receptor agonist 7-OH-DPAT inhibited NHE activity in renal tubules from 4- and 12-week-old SHR and 12-week-old WKY, but not in 4-week-old WKY. Considering that immortalized renal WKY and SHR cells were obtained from 4- to 8-week-old WKY and SHR animals, the present results suggest that differences in the ontogeny of dopamine D_3 receptors may occur between WKY and SHR animals.

The data presented here also show that NHE activity in SHR cells (immortalized and freshly isolated renal proximal tubules) were greater than in WKY cells. This is in agreement with previous reports while showing that NHE activity in renal proximal tubules from the SHR was significantly greater than in the WKY (Hayashi *et al*., 1997; LaPointe *et al*., 2002). Moreover, the difference in NHE activity between WKY and SHR renal tubules was more marked at 4 weeks than at 12 weeks. This and the view that the uncoupled inhibitory effect of D_1-like dopamine receptors on NHE3 activity in SHR precedes the development of hypertension (Li *et al*., 2001) may support the effect of D_3 dopamine receptor to an effort to overcome the abnormality in activity and regulation of NHE.

The low sensitivity of the NHE to inhibition by amiloride and EIPA correlated well with the IC_{50} values reported for the inhibition of NHE3 activity (Noel & Pouyssegur, 1995). Thus, this would agree with the fact that NHE3 isoforms predominate in rat renal proximal tubules (Biemesderfer *et al*., 1997; Karim *et al*., 1999). This suggestion is in line with the findings on the expression of NHE1 and NHE3. The increase in NHE activity in immortalized SHR cells might be explained by increases in NHE1 and NHE3. However, the increase in NHE activity in freshly isolated renal proximal tubules from the 12-week-old SHR appears to be dependent mainly on an increase in NHE3 activity, since the expression of NHE1 was actually reduced in SHR renal cortical membranes. Recently, however, a novel isoform of NHE, the EIPA-sensitive NHE8, was described in renal cortical brush border membranes (Goyal *et al*., 2003), but differences in its abundance between SHR and WKY, if any, have not been reported. It is unlikely that NHE being inhibited by 7-OH-DPAT in immortalized SHR cells corresponds to NHE1, since the 7-OH-DPAT-induced inhibition of NHE was insensitive to amiloride (10 μM). This would agree with the finding that NHE3 is more abundant than NHE1 in immortalized SHR cells and amiloride is more potent in immortalized SHR cells than in WKY cells. On the other hand, 7-OH-DPAT-induced inhibition of NHE in WKY freshly isolated renal proximal tubules could be related to the inhibition of NHE1 since this is more abundant in WKY than in SHR. If D_3 receptors also inhibit NHE1, which is located at the basolateral membrane, this should prevent and increase in intracellular sodium. This would explain why the D_3 receptors do not engender a natriuresis in the SHR (Ladines *et al*., 2001) and salt-sensitive Dahl rats (Luippold *et al*., 2001). Differences in NHE inhibition by D_3 receptors in immortalized cells and in proximal tubules may be related to maintenance of cell polarity in tubules, but not in cells grown on plastic supports.

In conclusion, the findings reported here show an increase in NHE activity in SHR renal proximal tubule immortalized cells, similar to that occurring in fresh isolated cells. Dopamine D_3 receptors coupled to a $G_{i/o}$ protein play a role in the handling of tubular Na^+, namely through inhibition of the NHE activity, this being of particular relevance in the SHR, which fail to respond to D_1-like dopamine receptor stimulation. Differences in the ontogeny of dopamine D_3 receptors may occur between WKY and SHR animals.

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