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Dopamine acutely decreases type 3 Na^+/H^+ exchanger activity in renal OK cells through the activation of protein kinases A and C signalling cascades

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Abstract

Dopamine D_1-mediated inhibition of Na^+,K^+-ATPase activity in opossum kidney (OK) cells involves the sequential activation of the adenylyl cyclase–protein kinase A (PKA) and the phospholipase C–protein kinase C (PKC) pathways [Am. J. Physiol. Renal Physiol. 282 (2002) F1084.]. The present study evaluated the signalling cascades involved in dopamine-mediated inhibition of Na^+/H^+ exchanger isoform 3 (NHE3) in OK cells. The transport kinetics displayed a simple Michaelis–Menten relationship for extracellular Na^+ of 25 ± 6 mM. Dopamine and the dopamine D_1-like receptor agonist SKF 38393 ((±)-1-phenyl-2,3,4,5-tetrahydro-(1*H*)-3-benzazepine-7,8-diol) inhibited NHE3 activity in a concentration-dependent manner; the dopamine D_2-like receptor agonist quinerolane was devoid of effect. The SKF 38393-mediated inhibition of NHE3 was prevented either by the dopamine D_1-like receptor antagonist SKF 83566 ((±)-7-Bromo-8-8-hydroxy-3 methyl-1-phenyl-2,3,4,5-tetrahydro-1*H*-3-benzazepine; 1 μM), overnight treatment with cholera toxin (500 ng/ml), the PKA antagonist H-89 (*N*-(2-[*p*-bromocinnamylamino]ethyl)-5 isoquinolinesulfonamide hydrochloride; 10 μM), the PKC antagonist chelerythrine (1 μM), or the phospholipase C inhibitor U-73,122 (1-(6-[(17β]-3-methoxyestra-1,3,5[10]-trien-17-yl) amino] hexyl)-1*H*-pyrrole-2,5-dione; 3 μM). In addition, dibutyril cAMP (dB-cAMP; 500 μM) was found to increase phospholipase C activity, both in membranes and in cytosol from OK cells; in contrast, phorbol-12,13-dibutyrate (PDB) (1 μM) did not have a significant effect on phospholipase C activity. Pre-treatment of OK cells with the anti-$G_s\alpha$ antibody, but not the anti-$G_{q/11}\alpha$ antibody, blunted the inhibitory effect of SKF 38393 on NHE3 activity. It is concluded that dopamine D_1-mediated inhibition of NHE3 in renal OK cells involves both adenylyl cyclase–PKA and the phospholipase C–PKC pathways, a mechanism similar to that described for Na^+,K^+-ATPase.

Keywords: Na^+/H^+ exchanger; Dopamine D_1-like receptor; Protein kinase; OK cell

1. Introduction

Dopamine is recognised as an important peripheral hormone modulating renal sodium excretion and blood pressure by its cell-to-cell actions within the kidney (Jose et al., 1992). Under conditions of moderate sodium loading, endogenous renal dopamine accounts for ~50% of sodium excreted (Hegde et al., 1989; Pelayo et al., 1983; Siragy et al., 1989). Regulation of sodium transport across the proximal tubules occurs through the involvement of two key proteins: the apical Na^+/H^+ exchanger (NHE), and the basolateral Na^+,K^+-ATPase (Aperia et al., 1987; Felder et al., 1990; Jose et al., 1992). These two membrane transporters have been identified as targets for the action of dopamine.

The activation of dopamine D_1-like receptors by dopamine and dopamine D_1-like agonists produces inhibition of NHE activity in the proximal segments of the nephron, as well as in brush-border membrane vesicle preparations (Felder et al., 1990; Gesek and Schoolwerth, 1990), although it is unclear whether the signal is transmitted via protein kinase A (PKA) and/or protein kinase C (PKC). In contrast to the well-documented role of dopamine D_1-like receptors in NHE regulation, activation of dopamine D_2-like receptors has been suggested not to affect NHE activity in the proximal tubule (Felder et al., 1990).

There are currently eight cloned mammalian NHEs, which differ from each other in tissue distribution, pharmacological properties, localisation and function (Goyal et al., 2003; Hayashi et al., 2002; Noel and Pouyssegur, 1995). All isoforms are expressed in renal tissues, with the exception of the NHE isoform type 5. The epithelial isoform of type 3 NHE (NHE3) is largely responsible for sodium and hydro-

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gen ion transport in the renal proximal convoluted tubule. The established renal opossum kidney (OK) cell line (Koyama et al., 1978) is a well-known in vitro model of the proximal tubular function, which expresses solely the apical NHE3 isoform (Azarani et al., 1995; Noel et al., 1996). This feature makes them a useful tool for the study of NHE3 regulation. NHE3 activity is acutely regulated by phosphorylation/dephosphorylation processes and membrane recycling in intact cells (Kurashima et al., 1997; Moe, 1999; Wiederkehr et al., 1999; Zhao et al., 1999). NHE3 can also be regulated by G proteins independent of cytosolic second messengers (Albrecht et al., 2000; Brunskill et al., 1992; Felder et al., 1993).

We have recently reported that dopamine-induced inhibition of Na^+,K^+-ATPase in OK cells is mediated by dopamine D_1-like receptors and involves the sequential activation of adenylyl cyclase–PKA and the phospholipase C–PKC systems (Gomes and Soares-da-Silva, 2002). In the present study, we examined the hypothesis whether dopamine also inhibits the apical NHE3 by both PKA and PKC pathways in the OK cell model system. Our results demonstrate that dopamine D_1-, but not dopamine D_2-like receptors, inhibit NHE3 activity via two signalling pathways that are sequentially activated, a mechanism similar to that previously described for Na^+,K^+-ATPase.

2. Methods

2.1. Cell culture

OK cells (ATCC 1840-HTB) were obtained from the American Type Culture Collection (Rockville, MD) and maintained in a humidified atmosphere of 5% CO_2–95% air at 37 °C. OK cells (clone OK_{HC}, passages 53–74; Gomes et al., 2002) were grown in Minimal Essential Medium (Sigma, St. Louis, MO, USA) supplemented with 100 U/ml penicillin G, 0.25 μg/ml amphotericin B, 100 μg/ml streptomycin (Sigma), 10% foetal bovine serum (Sigma) and 25 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanosulfonic acid (HEPES; Sigma). Once a week, the cells were dissociated by trypsinization, split 1:5 and cultured in plastic culture dishes with 21- or 55-cm^2 growth areas (Costar, Badhoevedorp, The Netherlands). The cell medium was changed every 2 days, and the cells reached confluence after 3–4 days of incubation. For 24 h prior to each experiment, the cells were maintained in foetal bovine serum-free medium. Experiments were generally performed 1–2 days after cells reached confluence and 5–7 days after the initial seeding; each cm^2 contained about 80–100 μg of cell protein. In some experiments, cells were treated overnight with agents known to interfere with signal transducing pathways, namely G proteins, such as cholera toxin (Sigma), pertussis toxin (Sigma) and specific antibodies raised against $G_s\alpha$ or $G_{q/11}\alpha$ proteins (Calbiochem, San Diego, CA). To minimise difficulties in the entry of the antibodies into the cell, anti-$G_s\alpha$ and anti-$G_{q/11}\alpha$ antibodies (1:500) were prepared in the presence of lipofectin (1%, v/v; Gibco, Grand Island, NY) and foetal bovine serum-free culture medium (Gomes and Soares-da-Silva, 2002). On the day of the experiment, culture medium containing the test agents was removed and the cells were washed with fresh medium and allowed to stabilise for at least 2 h before the start of experiments.

2.2. Intracellular pH measurement and NHE activity

Intracellular pH (pH_i) was measured as previously described (Gomes et al., 2001; Vieira-Coelho et al., 2001). At days 5–7 after seeding, cells cultured on 96 well plates (Costar) were incubated at 37 °C for 40 min with 5 μM of the acetoxymethyl ester of 2′,7′-bis(carboxyethyl)-5(6)-carboxyfluorescein (BCECF-AM). Cells were then washed twice with prewarmed dye-free modified Krebs' buffer before initiation of the fluorescence recordings. Cells were placed in the sample compartment of a dual-scanning microplate spectrofluorometer (Spectramax Gemini, Molecular Devices, Sunnyvale, USA), 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. The ratio of intracellular BCECF fluorescence at 490 and 440 nm was converted to pH_i values by comparison with values from an intracellular calibration curve using the nigericin (10 μM) and high-K^+ method (Gomes et al., 2001).

NHE activity was assayed as the initial rate of pH_i recovery after an acid load imposed by 20 mM NH_4Cl followed by 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 (Gomes et al., 2001). In these experiments, NaCl was replaced by an equimolar concentration of tetramethylammonium chloride (TMA). Test compounds were added to the extracellular fluid 25–30 min before the start of Na^+-dependent pH_i recovery. In some experiments, dopamine or dopamine agonists and antagonists were added to the extracellular fluid 10–60 min before the beginning of pH_i recovery.

2.3. Phospholipase C activity

Phospholipase C activity was assayed as previously described (Gomes and Soares-da-Silva, 2002). OK cells grown in six well culture clusters were incubated for 15 min at 37 °C with test compounds in Hanks' medium. Washing the cells with ice-cold Hanks' medium three times terminated the incubations. Subsequently, the cells were lysed by adding lysis buffer containing (in mM) 20 Tris–HCl, pH 7.4, 2 EDTA, 2 phenylmethylsulfonyl fluoride, 25 sodium pyrophosphate, 20 sodium fluoride, and 10 μg/ml each leupeptin and aprotinin. Thereafter, the cells were centrifuged at 4000 rpm for 20 min at 4 °C and the cytosol and membrane fractions separated for the assay of PLC activity. The cytosol and membranes were assayed for phospholipase C activity

using the Amplex Red phosphatidylcholine-specific phospholipase C assay kit (Molecular Probes, Eugene, OR), using a Spectramax Gemini dual-scanning fluorescence microplate reader (Molecular Devices). In brief, phospholipase C activity was monitored indirectly using 10-acetyl-3,7-dihydroxyphenoxazine (Amplex Red reagent), a sensitive fluorogenic probe for H_2O_2. Assays were performed in 96 well plates, with 200 μl reaction volume. First, phospholipase C converts the phosphatidylcholine (lecithin) substrate to form phosphocholine and diacylglycerol. After the action of alkaline phosphatase, which hydrolyses phosphocholine, choline is oxidized by choline oxidase to betaine and H_2O_2. Finally, H_2O_2 in the presence of horseradish peroxidase reacts with Amplex Red reagent in a 1:1 stoichiometry, to generate the highly fluorescent product, resorufin.

2.4. 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: $Y = V_{max} * X/(K_m + X)$; Y represents the NHE activity (pH units/s) and X the Na^+ concentration. Arithmetic means are given with S.E.M. or geometric means with 95% confidence values. Statistical analysis was done 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.

2.5. Drugs

Amiloride, chelerythrine chloride, cholera toxin, dibutyril cAMP, dopamine hydrochloride, forskolin, guanosine 5′-*O*-(3-thiotriphosphate) (GTPγS), H-89 (*N*-(2-[*p*-bromocinnamylamino]ethyl)-5 isoquinolinesulfonamide hydrochloride), PD 98059 (2-(2-amino-3-methoxyphenyl)-4*H*-1-benzopyran-4-one), pertussis toxin, phorbol-12,13-dibutyrate, SB 203580 (4-(4-Fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)-1*H*-imidazol), trypan blue and U-73,122 (1-(6-[(17β]-3-methoxyestra-1,3,5[10]-trien-17-yl) amino] hexyl)-1*H*-pyrrole-2,5-dione) were purchased from Sigma. Quinerolane hydrochloride, SKF 83566 ((±)-7-bromo-8-8-hydroxy-3 methyl-1-phenyl-2,3,4,5-tetrahydro-1*H*-3-benzazepine) and SKF 38393 ((±)-1-phenyl-2,3,4,5-tetrahydro-(1*H*)-3-benzazepine-7,8-diol) were obtained from Research Biochemicals International (Natick, USA). Acetoxymethyl ester of 2′,7′-bis(carboxyethyl)-5(6)-carboxyfluorescein (BCECF-AM), EIPA (5-(*N*-ethyl-*N*-isopropyl)-amiloride) and nigericin were obtained from Molecular Probes.

3. Results

In the present study, NHE activity was assayed in OK cells loaded with a pH-sensitive dye (BCECF), as the Na^+-dependent recovery of pH_i measured after an acid load imposed by 20 mM NH_4Cl followed by removal of Na^+ from the Krebs modified buffer solution, in the absence of CO_2/HCO_3. As shown in Fig. 1A, after acidification, OK cells showed a rapid alkalinization upon addition of 140 mM Na^+. This alkalinization process was inhibited by amiloride (1 mM) and by EIPA (10 μM), indicating that

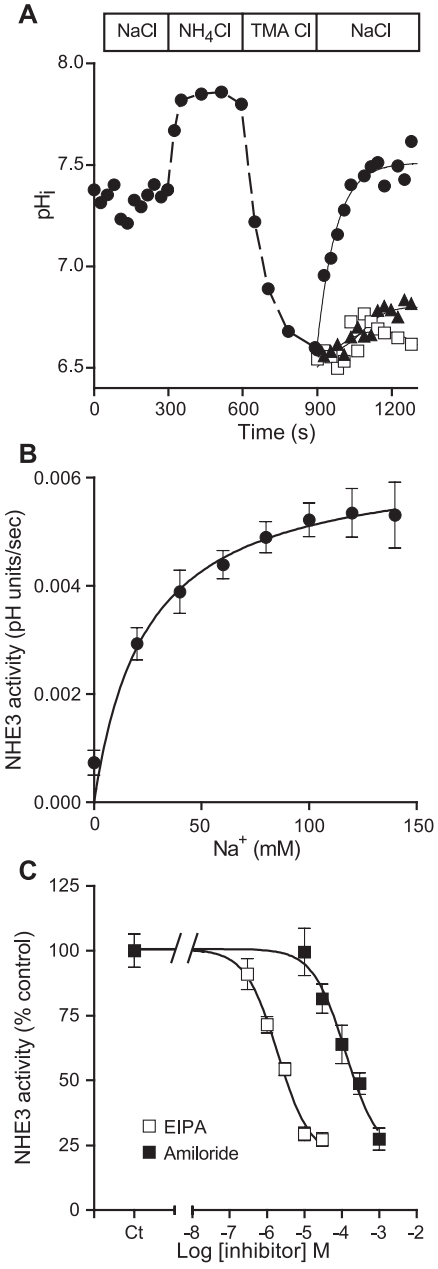

Fig. 1. (A) Composite tracing of a representative experiment demonstrating recovery of pH_i after an acid load imposed by an NH_4Cl pulse followed by sodium removal, in control conditions (●; Na^+ 140 mM) and in OK cells treated with amiloride (▲; 1 mM) or EIPA (□; 10 μM). (B) Na^+-dependence and (C) concentration-dependent inhibition of Na^+/H^+ exchanger isoform 3 (NHE3) activity by amiloride and EIPA in OK cells. Each point represents the mean of six to twelve experiments per group; vertical lines indicate S.E.M. Absolute Na^+-dependence recovery of pH_i in pH units/s was 0.0061 ± 0.0004 (n = 16).

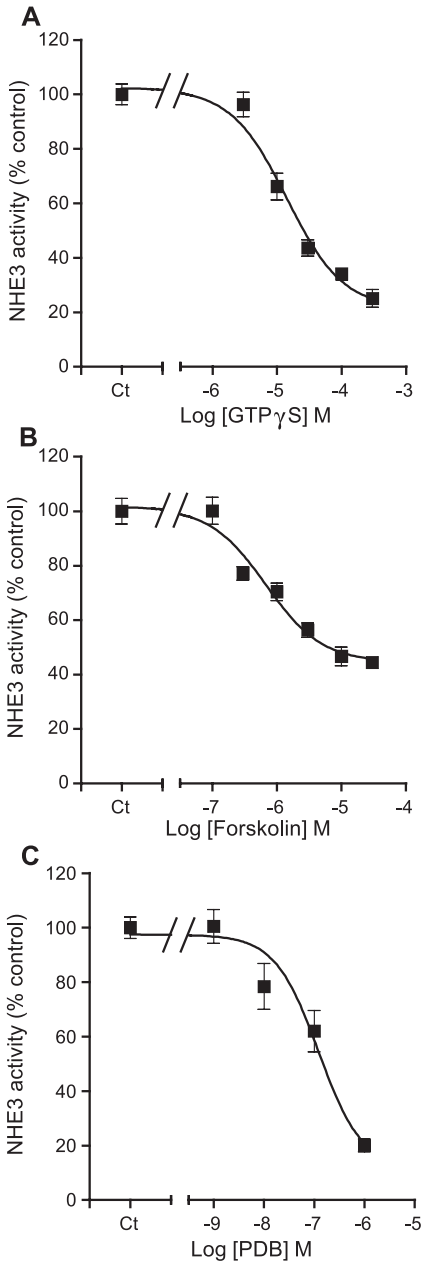

Fig. 2. Effect of (A) GTPγS, (B) forskolin and (C) PDB on NHE3 activity in OK cells. Each point represents the mean of six to eight experiments per group; vertical lines indicate S.E.M. Significantly different from control values (*$P<0.05$). Absolute Na^+-dependence recovery of pH_i in pH units/s was 0.0067 ± 0.0006 ($n=24$).

endogenous NHE in OK cells is an amiloride-inhibitable NHE. To define the kinetic activation of pH_i recovery by Na^+ in the apical medium, pH_i recovery was evaluated at increasing extracellular Na^+ concentrations (0 to 140 mM). Results shown in Fig. 1B indicate that the recovery of pH_i was clearly a Na^+-dependent process in OK cells. The Na^+ activation kinetics are well fit by the Michaelis–Menten equation, with a maximal velocity (V_{max}) of 0.0063 ± 0.0004 pH units/s and with a half-maximal transport rate (K_m) of 24.5 ± 6.3 mM Na^+. The sensitivity of NHE to inhibition by amiloride and EIPA was also evaluated. Fig. 1C shows inhibition of NHE activity in OK cells by both amiloride and EIPA, being EIPA considerably more potent than amiloride (IC_{50} values: amiloride, $IC_{50}=125$ [46,339] μM; EIPA, $IC_{50}=2$ [1,4] μM). Differences in sensitivity to inhibitors are in agreement with the observation that OK cells express mainly the epithelial NHE3 (Azarani et al., 1995; Noel et al., 1996).

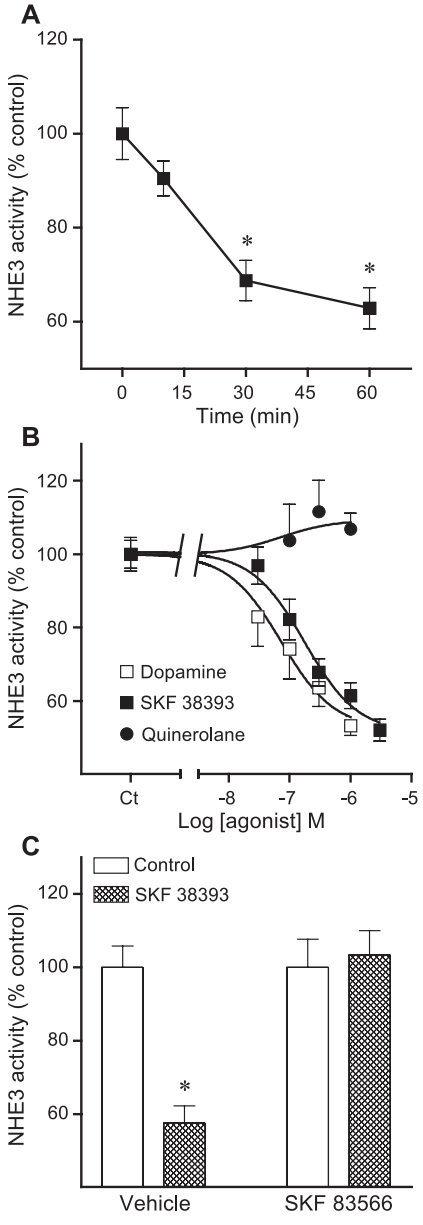

Fig. 3. (A) Time-dependence of dopamine (1 μM) on NHE3 activity in OK cells. (B) Concentration-dependence of dopamine, D_1-like agonist SKF 38393 and D_2-like agonist quinerolane on NHE3 activity in OK cells. (C) Effect of SKF 38393 (1 μM) in the absence and presence of the D_1-like antagonist SKF 38396 (1 μM). Symbols and columns represent the mean of five to twelve experiments per group; vertical lines indicate S.E.M. Significantly different from control values (*$P<0.05$). Absolute Na^+-dependence recovery of pH_i in pH units/s was 0.0058 ± 0.0003 ($n=40$).

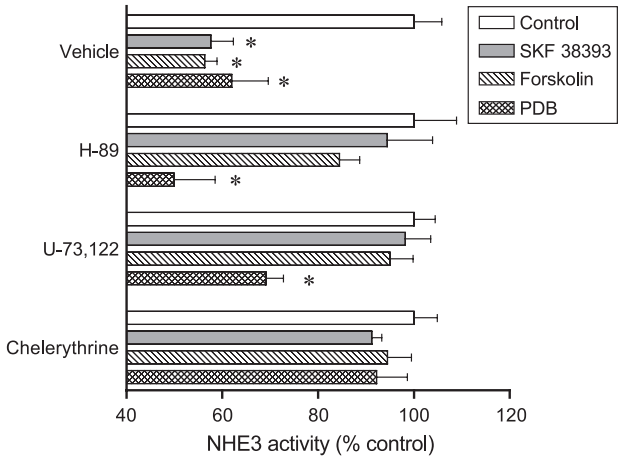

Fig. 4. Effect of PKA (H-89; 10 μM), phospholipase C (U-73,122; 3 μM) and PKC (chelerythrine; 1 μM) inhibitors on SKF 38393-, forskolin- and phorbol-12,13-dibutyrate (PDB)-mediated inhibition of NHE3 activity in OK cells. Columns represent the mean of eight experiments per group; vertical lines indicate S.E.M. Significantly different from corresponding control value (*$P < 0.05$). Absolute Na^+-dependence recovery of pH_i in pH units/s was 0.0063 ± 0.0005 ($n = 8$).

Earlier studies have demonstrated that pharmacological activation of G proteins and protein kinases A and C can lead to inhibition of NHE3 activity (Felder et al., 1993; Kurashima et al., 1997; Wiederkehr et al., 1999). To confirm whether these regulatory mechanisms are present and functional in OK cells, the effects of GTPγS, a nonhydrolyzable GTP analogue known for its role as a G protein activator, forskolin, a direct agonist of adenylyl cyclase, and phorbol-12,13-dibutyrate (PDB), a potent activator of PKC, were examined. Treatment of OK cells with increasing concentrations of GTPγS (Fig. 2A), forskolin (Fig. 2B) and PDB (Fig. 2C), effectively reduced NHE3 activity.

Next, we evaluated the effect of dopamine and selective dopamine receptor agonists on NHE3 activity. Fig. 3A shows the time dependency of dopamine (1 μM) on NHE3 activity. In our experimental conditions, dopamine-induced NHE3 inhibition reached maximal levels after 30-min incubation. Thus, in all subsequent experiments, cells were exposed to dopamine or selective agonists for ~ 30 min. As shown in Fig. 3B, both dopamine and the selective D_1-like receptor agonist SKF 38393 were found to attenuate, in a concentration-dependent manner, the Na^+-dependent pH_i recovery in OK cells; by contrast, the selective D_2-like receptor agonist quinerolane was devoid of effect. The inhibitory effect of SKF 38393 (1 μM) upon NHE3 activity was blocked by the D_1-like receptor antagonist SKF 83566 (1 μM) (Fig. 3B). Altogether, these results suggest that the dopamine-induced inhibition of NHE3 activity in OK cells is via D_1-like receptors.

The intracellular signalling pathways involved in D_1-like receptor-mediated inhibition of NHE activity may include activation of PKA and/or PKC pathways (Hussain and Lokhandwala, 1998; Jose et al., 1998). To address this point, OK cells were treated with selective antagonists of PKA (H-89) or PKC (chelerythrine) (Azarani et al., 1995). As shown in Fig. 4, H-89 (10 μM) prevented the inhibitory effects of both SKF 38393 (1 μM) and forskolin (10 μM); however, PDB was still able to inhibit NHE3 activity. Chelerythrine (1 μM) antagonized the effects of SKF 38393, forskolin and PDB. These results suggest that stimulation of D_1-like receptors may lead to simultaneous activation of both PKA and PKC transduction pathways with a common point in the cascade of events, since both H-89 and chelerythrine completely antagonised the effect of the D_1-like agonist. The ability of PDB to inhibit NHE3 activity in conditions of PKA inhibition and the failure of SKF 38393 and forskolin to reduce NHE3 activity in conditions of PKC inhibition suggests that PKA may be activated prior to PKC activation. One possible sequence of events could be the activation of phospholipase C by PKA, prior to activation of PKC. To validate this hypothesis, we tested the phospholipase C inhibitor U-73,122 (Bleasdale et

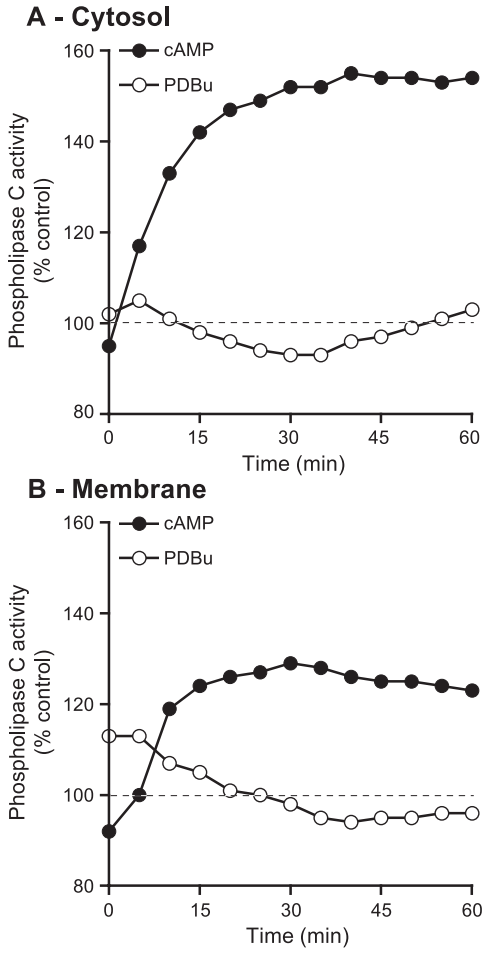

Fig. 5. Effect of dibutyryl-cAMP (500 μM) and phorbol-12,13-dibutyrate (PDB; 1 μM) on phospholipase C activity in (A) membrane and (B) cytosolic fractions of OK cells. Cells were stimulated with test compounds for 15 min and then phospholipase C activity measured as described in Methods. Results are expressed as percentage of control (dotted line). Symbols represent the mean of three experiments per group. Absolute phospholipase C activity was 25 ± 4 pmol/mg protein ($n = 24$).

al., 1990) upon the effect of SKF 38393, forskolin and PDB. As indicated in Fig. 4, U-73,122 (3 μM) was able to prevent the inhibitory effects of both SKF 38393 (1 μM) and forskolin (10 μM) upon NHE3 activity. However, the phospholipase C inhibitor U-73,122 (3 μM) did not affect the inhibitory effect of 100 nM PDB. To further prove the view that phospholipase C activation may occur downstream of PKA activation, complementary studies on phospholipase C activity in OK cells were performed. Results shown in Fig. 5 show that dibutyryl cAMP (500 μM), but not PDB (1 μM), increased both cytosolic and membrane phospholipase C activity in OK cells.

Recent reports have proposed the regulation of renal NHE3 in a mitogen-activated protein kinase (MAPK)-dependent manner (Liu and Gesek, 2001; Tsuganezawa et al., 2002). To test whether this signalling pathway plays a role in D_1-like receptor-mediated inhibition of NHE3 activity, OK cells were incubated overnight with specific inhibitors of either p38 MAPK (SB 203580) or MEK1 (PD 98059), the upstream activator of the extracellular signal-regulated kinase (ERK). As shown in Fig. 6, the pharmacological inhibitors of MAPK failed to prevent the inhibitory effect of SKF 38393. Thus, these results indicate that the ERK/MAPK pathway is not involved in D_1-like receptor-mediated regulation of NHE3 in OK cells.

Having shown that D_1-like receptors activate both PKA and PKC pathways, we next evaluated the involvement of G proteins in the regulation of NHE3 in OK cells. Cholera toxin was used to maximally activate the GTP-binding protein (G protein) $G_s\alpha$ and uncouple the D_1-like receptor from $G_s\alpha$ (Liu et al., 1992). Overnight treatment of OK cells with cholera toxin (500 ng/ml) abolished the effect of SKF 38393 (1 μM) on NHE3 activity (Fig. 7). In contrast, preincubation (overnight) with pertussis toxin, whose main function is the ribosylation of α-subunits of G_i and G_o proteins (West et al., 1985), did not block the inhibitory effect of the D_1-like receptor agonist (Fig. 7). These results suggest that D_1-like receptors stimulated by SKF 38393 are coupled to cholera toxin-sensitive G proteins of the G_s class. To further elucidate the coupling of D_1-like receptors to G proteins in OK cells, additional studies were performed in cells treated overnight with antibodies raised against rat $G_s\alpha$ and $G_{q/11}\alpha$ proteins. Antibodies were complexed with liposomes in order to facilitate delivery to the cytosol. As shown in Fig. 8, the inhibitory effect of SKF 38393 upon NHE3 was abolished in cells treated with the anti-$G_s\alpha$ antibody, but not in cells treated with the anti-$G_{q/11}\alpha$ antibody. These results agree with the view that D_1-like receptors in OK cells are coupled to adenylyl cyclase via a $G_s\alpha$ type of G protein and subsequent activation of phospholipase C-PKC systems does not involve a $G_{q/11}\alpha$ type of G protein, but most probably results from activation of phospholipase C by PKA.

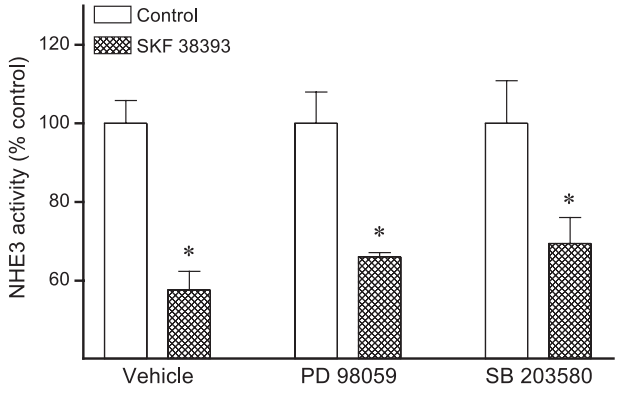

Fig. 6. Effect of SKF 38393 (1 μM) on NHE3 activity in OK cells in the absence and the presence of MAPK inhibitors. Cells were incubated overnight with vehicle, PD 98059 (10 μM) or SB 203580 (10 μM). Columns represent the mean of eight experiments per group; vertical lines indicate S.E.M. Significantly different from corresponding control value (*$P<0.05$). Absolute Na^+-dependence recovery of pH_i in pH units/s was 0.0063 ± 0.0007 ($n=8$).

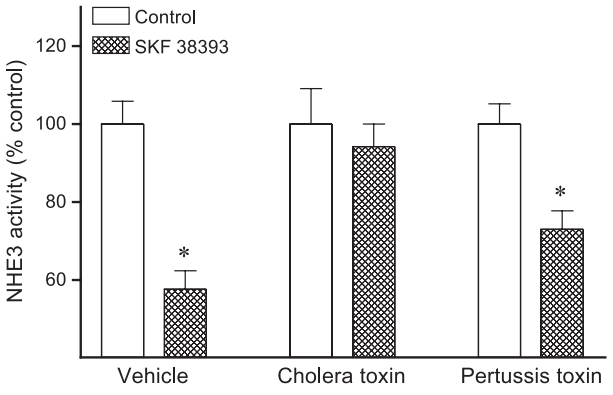

Fig. 7. Effect of SKF 38393 (1 μM) on NHE3 activity in OK cells in the absence and the presence of cholera toxin or pertussis toxin. Cells were incubated overnight with vehicle, cholera toxin (500 ng/ml) or pertussis toxin (100 ng/ml). Columns represent the mean of five to nine experiments per group; vertical lines indicate S.E.M. Significantly different from corresponding control value (*$P<0.05$). Absolute Na^+-dependence recovery of pH_i in pH units/s was 0.0060 ± 0.0008 ($n=8$).

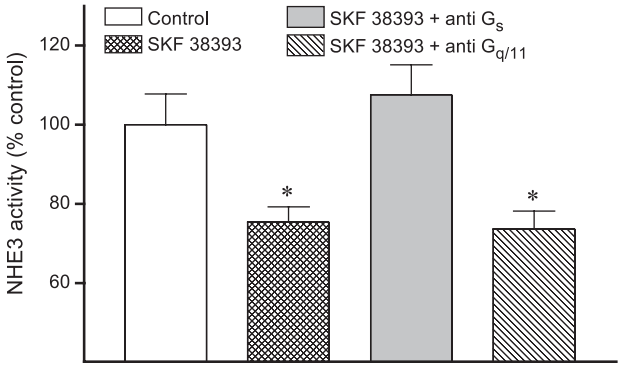

Fig. 8. NHE3 activity in OK cells after overnight treatment with lipofectin plus vehicle or specific antibodies raised against $G_s\alpha$ or $G_{q/11}\alpha$ proteins, followed by short-term exposure to SKF 38393 (1 μM). Columns represent means of nine to twelve experiments per group and vertical lines show S.E.M. Significantly different from control value (*$P<0.05$). Absolute Na^+-dependence recovery of pH_i in pH units/s was 0.0062 ± 0.0003 ($n=8$).

4. Discussion

Previous studies have established that the natriuretic effect induced by dopamine results from inhibition of the activities of the major Na^+ transporters in the proximal tubules, namely the apical NHE and the basolateral Na^+,K^+-ATPase (Aperia et al., 1987; Felder et al., 1990; Jose et al., 1992). We undertook the present study to examine the intracellular pathways triggered by stimulation of D_1-like receptors downstream to NHE3 inhibition in monolayers of opossum kidney cells. Our results indicate that inhibition of NHE3 activity by D_1-like receptors involves both adenylyl cyclase–PKA and the phospholipase C–PKC systems, a mechanism similar to that previously described for Na^+,K^+-ATPase (Gomes and Soares-da-Silva, 2002). Furthermore, D_1-like receptors in OK cells are coupled to $G_s\alpha$, but not to $G_{q/11}\alpha$ proteins, and the chain of molecular events leading to NHE3 inhibition begins with activation of the adenylyl cyclase-PKA system followed by activation of the phospholipase C-PKC system, being phospholipase C an effector protein for PKA.

An important component of acute regulation of NHE3 is by phosphorylation/dephosphorylation processes (Kurashima et al., 1997; Moe, 1999; Wiederkehr et al., 1999; Zhao et al., 1999). Regulation of NHE3 by PKA has been widely reported in the literature (Kurashima et al., 1997; Wiederkehr et al., 2001; Zhao et al., 1999). As it relates to PKC, the results are less consensual. Some authors observed a stimulatory effect of NHE3 activity during PKC activation (Karim et al., 1999; Wiederkehr et al., 1999), whereas others observed acute inhibition of NHE3 activity during PKC activation (Di Sole et al., 2003; Donowitz et al., 2000; Lee-Kwon et al., 2003; Tse et al., 1993). Differences in cellular preparations (with endogenous versus transfected NHE3) and differences in NHE assay methodologies may account for discrepant results. In the present study, both forskolin and PDB were able to inhibit NHE3 activity to the same extent, these effects were prevented by specific inhibitors of PKA (H-89) and PKC (chelerythrine). In addition, GTPγS inhibits NHE3 activity, presumably by stimulating G proteins. The present studies indicate that G protein activation, PKA and PKC pathways acutely regulate NHE3 in OK cells.

Dopamine, at concentrations in the nM–low μM range, activates dopamine receptors. At higher doses, dopamine also activates β- and α-adrenoceptors (van Veldhuisen et al., 1992). It has been shown that UK-14304, an α_2-adrenoceptor agonist, stimulates NHE3 activity via α_2-adrenergic receptors/$G_i\alpha$ in intact opossum kidney cells (Clarke et al., 1990). Therefore, the regulation of NHE by dopamine should be demonstrated at concentrations within the physiological range to be considered crucial in the modulation of renal sodium handling. For this reason, we have studied the effect of dopamine upon NHE3 at concentrations lower than those used in other studies (Wiederkehr et al., 2001). The rationale for the use of lower concentrations of dopamine is based on the fact that OK cells express endogenous α_2-adrenergic receptors (Blaxall et al., 1994), which could be activated by higher concentrations of dopamine, and thus produce a stimulatory, rather than an inhibitory, effect upon NHE3 activity. In our hands, physiological levels of dopamine inhibited NHE3 activity, in a time- and concentration-dependent manner. Several lines of evidence suggest that the inhibitory effect of dopamine upon NHE3 activity in OK cells is mediated by D_1-like receptors. First, the D_1-like receptor agonist SKF 38393 produced a concentration-dependent inhibition of NHE3. Second, the D_2-like receptor agonist quinerolane was ineffective in altering NHE3 activity. Finally, D_1-like blockade prevented the effect of SKF 38393 on NHE3 activity. This is consistent with previous reports showing that the regulation of tubule sodium reabsorption by dopamine is primarily mediated by the D_1-like receptor family (Hussain and Lokhandwala, 1998; Jose et al., 2000).

Earlier reports had implicated the involvement of PKA and/or PKC pathways in the dopamine-induced inhibition of NHE activity in renal cells (Hussain and Lokhandwala, 1998; Jose et al., 1998). In our studies, selective inhibition of PKA and PKC pathways with H-89 and chelerythrine, respectively, prevented the decrease in NHE3 activity by the D_1-like receptor agonist. This suggests that both kinases participate in the signal transduction pathway following D_1-like receptor activation, but does not prove that the signal transmitted by D_1-like receptors may lead to independent activation of PKA and PKC pathways. One possible interpretation considers a single sequence of events, with PKA activation prior to PKC activation in the cascade downstream to stimulation of D_1-like receptors. This view is supported by the finding that the phospholipase C inhibitor U-73,122 prevented the inhibitory effects of both forskolin and SKF 38393 on NHE3 activity, but not those of PDB. Moreover, dibutyryl-cAMP, but not PDB, produced a significant stimulation of phospholipase C activity both in membrane and cytosol preparations from OK cells. Taken together, these results strongly suggest that downstream D_1-like dopamine receptor activation to inhibition of NHE3 activity in OK cells there is a chain of events comprising adenyly cyclase-PKA activation, followed by activation of phospholipase C by PKA, the product of which, diacylglycerol, stimulates PKC. Our results contrast with previous reports showing that the dopamine-induced inhibition of NHE3 activity in OK cells is mediated by PKA and not by PKC pathways (Wiederkehr et al., 2001). The discrepancies in the involvement of PKC pathways may be due to a variability in substrains of OK cells in various laboratories (Allon and Parris, 1993; Cole et al., 1989; Gomes et al., 2002), as well as on the culture conditions.

To our knowledge, this chain of events constitutes a new signalling pathway, downstream D_1-like dopamine receptor activation leading to inhibition of NHE3 activity. Other studies (Bertorello and Aperia, 1990; Wiederkehr et al., 2001) have reported on complex processes leading to inhibition of Na^+,K^+-ATPase and NHE3 activities, namely,

the requirement of simultaneous activation of both D_1-like and D_2-like receptors. Although the OK cells are endowed with both D_1-like and D_2-like receptors, it is unlikely that D_2-like receptors are involved in the generation of responses leading to direct inhibition of Na^+,K^+-ATPase and NHE3 activities. In fact, SKF 38393 is a rather selective D_1-like receptor agonist, the effects of which have been shown, in the OK cell line, to be insensitive to the selective D_2-like receptor antagonist *S*-sulpiride (Gomes et al., 2001). Another example in which the involvement of both PKA and PKC activation was observed downstream of dopamine receptor activation is that of LTK cells stably transfected with the rat D_1 receptor cDNA (Yu et al., 1996). Yu et al. (1996) showed that the D_1-mediated stimulation of phospholipase C occurred as a result of PKA activation via stimulation of PKC. This model contrasts with our proposal in OK cells (Gomes and Soares-da-Silva, 2002), the main arguments being the lack of involvement of the $G_{q/11}$ type of G protein and the finding that inhibition of phospholipase C by U-73122 failed to prevent inhibition of NHE3 activity (this study) and Na^+,K^+-ATPase activity (Gomes and Soares-da-Silva, 2002) by PDB. Dual coupling to adenylyl cyclase and phospholipase C has been reported (Pfister et al., 1999) in OK cells for parathyroid hormone. However, it is likely that parathyroid hormone signal transduction via a cAMP-dependent pathway does not involve stimulation of phospholipase C (Pfister et al., 1999). This has been also observed (Fargin et al., 1991; Force et al., 1992; Francesconi and Duvoisin, 1998; Kuhn et al., 1996) for other types of receptors, in different tissues, in which dual coupling to adenylyl cyclase and phospholipase C generally involves independent pathways.

Another point we have addressed concerns the involvement of G proteins on the signal transduction activated by D_1-like receptors. The results from this study provide direct evidence that D_1-like receptors are coupled to G proteins of the $G_s\alpha$ class. Two observations support this view. First, the finding that inhibition of NHE3 activity by SKF 38393 was abolished by overnight treatment of OK cells with cholera toxin, but not with pertussis toxin. In a second series of experiments, we used antibodies raised against the carboxy terminal of $G_s\alpha$ and $G_{q/11}\alpha$ subunits to block interactions of G proteins with D_1-like receptors. Under these conditions, inhibition of NHE3 by the D_1-like agonist was abolished in cells treated with anti-$G_s\alpha$, but not with anti-$G_{q/11}\alpha$ antibodies.

In conclusion, transduction mechanisms triggered by activation of D_1-like receptors in OK cells involve the activation of both PKA and PKC pathways in a single sequence of events with PKA activation prior to PKC activation, which most likely includes phosphorylation of phospholipase C by PKA. The results from this study provide direct evidence that inhibition of NHE3 activity by dopamine in OK cells involves the activation of D_1-like dopamine receptors and a G protein of the $G_s\alpha$ class positively coupled to adenylyl cyclase. However, it remains to be determined whether the signalling circuitry described here is specific to this cell line, or if it represents the general mechanism of signal transduction for D_1-like receptors.

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References

Albrecht, F.E., Xu, J., Moe, O.W., Hopfer, U., Simonds, W.F., Orlowski, J., Jose, P.A., 2000. Regulation of NHE3 activity by G protein subunits in renal brush-border membranes. Am. J. Physiol., Regul. Integr. Comp. Physiol. 278, R1064–R1073.

Allon, M., Parris, M., 1993. Calcitriol stimulates Na^+–Pi cotransport in a subclone of opossum kidney cells (OK-7A) by a genomic mechanism. Am. J. Physiol. 264, F404–F410.

Aperia, A., Bertorello, A., Seri, I., 1987. Dopamine causes inhibition of Na^+–K^+-ATPase activity in rat proximal convoluted tubule segments. Am. J. Physiol. 252, F39–F45.

Azarani, A., Goltzman, D., Orlowski, J., 1995. Parathyroid hormone and parathyroid hormone-related peptide inhibit the apical Na^+/H^+ exchanger NHE-3 isoform in renal cells (OK) via a dual signaling cascade involving protein kinase A and C. J. Biol. Chem. 270, 20004–20010.

Bertorello, A., Aperia, A., 1990. Inhibition of proximal tubule Na^+–K^+-ATPase activity requires simultaneous activation of DA_1 and DA_2 receptors. Am. J. Physiol. 259, F924–F928.

Blaxall, H.S., Cerutis, D.R., Hass, N.A., Iversen, L.J., Bylund, D.B., 1994. Cloning and expression of the alpha 2C-adrenergic receptor from the OK cell line. Mol. Pharmacol. 45, 176–181.

Bleasdale, J.E., Thakur, N.R., Gremban, R.S., Bundy, G.L., Fitzpatrick, F.A., Smith, R.J., Bunting, S., 1990. Selective inhibition of receptor-coupled phospholipase C-dependent processes in human platelets and polymorphonuclear neutrophils. J. Pharmacol. Exp. Ther. 255, 756–768.

Brunskill, N.J., Morrissey, J.J., Klahr, S., 1992. G-protein stimulation inhibits amiloride-sensitive Na/H exchange independently of cyclic AMP. Kidney Int. 42, 11–17.

Clarke, J.D., Cragoe Jr., E.J., Limbird, L.E., 1990. Alpha 2-adrenergic receptors regulate Na^+–H^+ exchange via a cAMP-dependent mechanism. Am. J. Physiol. 259, F977–F985.

Cole, J.A., Forte, L.R., Krause, W.J., Thorne, P.K., 1989. Clonal sublines that are morphologically and functionally distinct from parental OK cells. Am. J. Physiol. 256, F672–F679.

Di Sole, F., Cerull, R., Petzke, S., Casavola, V., Burckhardt, G., Helmle-Kolb, C., 2003. Bimodal acute effects of A1 adenosine receptor activation on Na^+/H^+ exchanger 3 in opossum kidney cells. J. Am. Soc. Nephrol. 14, 1720–1730.

Donowitz, M., Janecki, A., Akhter, S., Cavet, M.E., Sanchez, F., Lamprecht, G., Zizak, M., Kwon, W.L., Khurana, S., Yun, C.H., Tse, C.M., 2000. Short-term regulation of NHE3 by EGF and protein kinase C but not protein kinase A involves vesicle trafficking in epithelial cells and fibroblasts. Ann. N. Y. Acad. Sci. 915, 30–42.

Fargin, A., Yamamoto, K., Cotecchia, S., Goldsmith, P.K., Spiegel, A.M., Lapetina, E.G., Caron, M.G., Lefkowitz, R.J., 1991. Dual coupling of the cloned 5-HT_{1A} receptor to both adenylyl cyclase and phospholipase C is mediated via the same G_i protein. Cell Signal. 3, 547–557.

Felder, C.C., Campbell, T., Albrecht, F., Jose, P.A., 1990. Dopamine inhibits Na^+–H^+ exchanger activity in renal BBMV by stimulation of adenylate cyclase. Am. J. Physiol. 259, F297–F303.

Felder, C.C., Albrecht, F.E., Campbell, T., Eisner, G.M., Jose, P.A., 1993. cAMP-independent, G protein-linked inhibition of Na^+/H^+ exchange in

renal brush border by D_1 dopamine agonists. Am. J. Physiol. 264, F1032–F1037.

Force, T., Bonventre, J.V., Flannery, M.R., Gorn, A.H., Yamin, M., Goldring, S.R., 1992. A cloned porcine renal calcitonin receptor couples to adenylyl cyclase and phospholipase C. Am. J. Physiol., Renal Fluid Electrolyte Physiol. 262, F1110–F1115.

Francesconi, A., Duvoisin, R.M., 1998. Role of the second and third intracellular loops of metabotropic glutamate receptors in mediating dual signal transduction activation. J. Biol. Chem. 273, 5615–5624.

Gesek, F.A., Schoolwerth, A.C., 1990. Hormonal interactions with the proximal Na^+–H^+ exchanger. Am. J. Physiol. 258, F514–F521.

Gomes, P., SoaresdaSilva, P., 2002. Role of cAMP-PKA-PLC signaling cascade on dopamine-induced PKC-mediated inhibition of renal Na^+–K^+-ATPase activity. Am. J. Physiol., Renal Physiol. 282, F1084–F1096.

Gomes, P., Vieira-Coelho, M.A., Soares-da-Silva, P., 2001. Ouabain-insensitive acidification by dopamine in renal OK cells: primary control of the Na^+/H^+ exchanger. Am. J. Physiol., Regul. Integr. Comp. Physiol. 281, R10–R18.

Gomes, P., Xu, J., Serrao, P., Doria, S., Jose, P.A., Soares-da-Silva, P., 2002. Expression and function of sodium transporters in two opossum kidney cell clonal sublines. Am. J. Physiol., Renal Physiol. 283, F73–F85.

Goyal, S., Vanden Heuvel, G., Aronson, P.S., 2003. Renal expression of novel Na^+/H^+ exchanger isoform NHE8. Am. J. Physiol., Renal Physiol. 84, F467–F473.

Hayashi, H., Szaszi, K., Grinstein, S., 2002. Multiple modes of regulation of Na^+/H^+ exchangers. Ann. N. Y. Acad. Sci. 976, 248–258.

Hegde, S.S., Jadhav, A.L., Lokhandwala, M.F., 1989. Role of kidney dopamine in the natriuretic response to volume expansion in rats. Hypertension 13, 828–834.

Hussain, T., Lokhandwala, M.F., 1998. Renal dopamine receptor function in hypertension. Hypertension 32, 187–197.

Jose, P.A., Raymond, J.R., Bates, M.D., Aperia, A., Felder, R.A., Carey, R.M., 1992. The renal dopamine receptors. J. Am. Soc. Nephrol. 2, 1265–1278.

Jose, P.A., Eisner, G.M., Felder, R.A., 1998. Renal dopamine receptors in health and hypertension. Pharmacol. Ther. 80, 149–182.

Jose, P.A., Eisner, G.M., Felder, R.A., 2000. Renal dopamine and sodium homeostasis. Curr. Hypertens. Rep. 2, 174–183.

Karim, Z.G., Chambrey, R., Chalumeau, C., Defontaine, N., Warnock, D.G., Paillard, M., Poggioli, J., 1999. Regulation by PKC isoforms of Na^+/H^+ exchanger in luminal membrane vesicles isolated from cortical tubules. Am. J. Physiol. 277, F773–F778.

Koyama, H., Goodpasture, C., Miller, M.M., Teplitz, R.L., Riggs, A.D., 1978. Establishment and characterization of a cell line from the American opossum (*Didelphys virginiana*). In Vitro 14, 239–246.

Kuhn, B., Schmid, A., Harteneck, C., Gudermann, T., Schultz, G., 1996. G proteins of the Gq family couple the H2 histamine receptor to phospholipase C. Mol. Endocrinol. 10, 1697–1707.

Kurashima, K., Yu, F.H., Cabado, A.G., Szabo, E.Z., Grinstein, S., Orlowski, J., 1997. Identification of sites required for down-regulation of Na^+/H^+ exchanger NHE3 activity by cAMP-dependent protein kinase. phosphorylation-dependent and -independent mechanisms. J. Biol. Chem. 272, 28672–28679.

LeeKwon, W., Kim, J.H., Choi, J.W., Kawano, K., Cha, B., Dartt, D.A., Zoukhri, D., Donowitz, M., 2003. Ca^{2+}-dependent inhibition of NHE3 requires PKC alpha which binds to E3KARP to decrease surface NHE3 containing plasma membrane complexes. Am. J. Physiol., Cell Physiol. 285, C1527–C1536.

Liu, F., Gesek, F.A., 2001. $Alpha_1$-Adrenergic receptors activate NHE1 and NHE3 through distinct signaling pathways in epithelial cells. Am. J. Physiol., Renal Physiol. 280, F415–F425.

Liu, Y.F., Civelli, O., Zhou, Q.Y., Albert, P.R., 1992. Cholera toxin-sensitive 3′,5′-cyclic adenosine monophosphate and calcium signals of the human dopamine-D_1 receptor: selective potentiation by protein kinase A. Mol. Endocrinol. 6, 1815–1824.

Moe, O.W., 1999. Acute regulation of proximal tubule apical membrane Na/H exchanger NHE-3: role of phosphorylation, protein trafficking, and regulatory factors. J. Am. Soc. Nephrol. 10, 2412–2425.

Noel, J., Pouyssegur, J., 1995. Hormonal regulation, pharmacology, and membrane sorting of vertebrate Na^+/H^+ exchanger isoforms. Am. J. Physiol. 268, C283–C296.

Noel, J., Roux, D., Pouyssegur, J., 1996. Differential localization of Na^+/H^+ exchanger isoforms (NHE1 and NHE3) in polarized epithelial cell lines. J. Cell Sci. 109, 929–939.

Pelayo, J.C., Fildes, R.D., Eisner, G.M., Jose, P.A., 1983. Effects of dopamine blockade on renal sodium excretion. Am. J. Physiol. 245, F247–F253.

Pfister, M.F., Forgo, J., Ziegler, U., Biber, J., Murer, H., 1999. cAMP-dependent and -independent downregulation of type II Na–Pi cotransporters by PTH. Am. J. Physiol., Renal Physiol. 276, F720–F725.

Siragy, H.M., Felder, R.A., Howell, N.L., Chevalier, R.L., Peach, M.J., Carey, R.M., 1989. Evidence that intrarenal dopamine acts as a paracrine substance at the renal tubule. Am. J. Physiol. 257, F469–F477.

Tse, C.M., Levine, S.A., Yun, C.H., Brant, S.R., Pouyssegur, J., Montrose, M.H., Donowitz, M., 1993. Functional characteristics of a cloned epithelial Na^+/H^+ exchanger (NHE3): resistance to amiloride and inhibition by protein kinase C. Proc. Natl. Acad. Sci. U. S. A. 90, 9110–9114.

Tsuganezawa, H., Sato, S., Yamaji, Y., Preisig, P.A., Moe, O.W., Alpern, R.J., 2002. Role of c-SRC and ERK in acid-induced activation of NHE3. Kidney Int. 62, 41–50.

van Veldhuisen, D.J., Girbes, A.R., de Graeff, P.A., Lie, K.I., 1992. Effects of dopaminergic agents on cardiac and renal function in normal man and in patients with congestive heart failure. Int. J. Cardiol. 37, 293–300.

Vieira-Coelho, M.A., Gomes, P., Serrão, M.P., Soares-da-Silva, P., 2001. D_1-like dopamine receptor activation and natriuresis by nitrocatechol COMT inhibitors. Kidney Int. 59, 1683–1694.

West Jr., R.E., Moss, J., Vaughan, M., Liu, T., Liu, T.Y., 1985. Pertussis toxin-catalyzed ADP-ribosylation of transducin. Cysteine 347 is the ADP-ribose acceptor site. J. Biol. Chem. 260, 14428–14430.

Wiederkehr, M.R., Zhao, H., Moe, O.W., 1999. Acute regulation of Na/H exchanger NHE3 activity by protein kinase C: role of NHE3 phosphorylation. Am. J. Physiol. 276, C1205–C1217.

Wiederkehr, M.R., Di Sole, F., Collazo, R., Quinones, H., Fan, L., Murer, H., Helmle-Kolb, C., Moe, O.W., 2001. Characterization of acute inhibition of Na/H exchanger NHE-3 by dopamine in opossum kidney cells. Kidney Int. 59, 197–209.

Yu, P.Y., Eisner, G.M., Yamaguchi, I., Mouradian, M.M., Felder, R.A., Jose, P.A., 1996. Dopamine D_{1A} receptor regulation of phospholipase C isoform. J. Biol. Chem. 271, 19503–19508.

Zhao, H., Wiederkehr, M.R., Fan, L., Collazo, R.L., Crowder, L.A., Moe, O.W., 1999. Acute inhibition of Na/H exchanger NHE-3 by cAMP. Role of protein kinase a and NHE-3 phosphoserines 552 and 605. J. Biol. Chem. 274, 3978–3987.