G_1_α_3 protein-coupled dopamine D_3 receptor-mediated inhibition of renal NHE3 activity in SHR proximal tubular cells is a PLC-PKC-mediated event

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Pedrosa, Rui, Pedro Gomes, Ulrich Hopfer, Pedro A. Jose, and Patricio Soares-da-Silva. G_1_α_3 protein-coupled dopamine D_3 receptor-mediated inhibition of renal NHE3 activity in SHR proximal tubular cells is a PLC-PKC-mediated event. Am J Physiol Renal Physiol 287: F1059–F1066, 2004. First published July 20, 2004; doi:10.1152/ajprenal.00139.2004.—This study evaluated the transduction pathway associated with type 3 Na^+/H^+ exchanger (NHE3) activity-induced inhibition during dopamine D_3 receptor activation in immortalized renal proximal tubular epithelial cells from the spontaneously hypertensive rat. The dopamine D_3 receptor agonist 7-OH-DPAT decreased NHE3 activity, which was prevented by the D_2-like receptor antagonist S-sulpiride, pertussis toxin (PTX; overnight treatment), and the PKC inhibitor chelerythrine, but not by cholinergic toxin (overnight treatment), the MAPK inhibitor PD-09059, or the p38 inhibitor SB-203580. The PKA inhibitor H-89 abolished the inhibitory effects of forskolin on NHE3 activity, but not that of 7-OH-DPAT. The phospholipase C (PLC) inhibitor U-73122 prevented the inhibitory effects of forskolin on NHE3 activity, but not that of 7-OH-DPAT. The 7-OH-DPAT-induced decrease in NHE3 activity was abolished in cells treated overnight with the anti-G_1_α_3 antibody, but not in cells treated with antibodies against G_1_α_1, G_1_α_2, G_1_α_5, and G_1_α_1_2_1_2 proteins. The calcium ionophore A-23187 and the Ca^2+ -ATPase inhibitor thapsigargin increased intracellular Ca^2+ but did not affect NHE3 activity. However, the inhibitory effects of PDBu and 7-OH-DPAT on NHE3 activity were completely abolished by A-23287 and thapsigargin. It is concluded that inhibition of NHE3 activity by dopamine D_3 receptors coupled to G_1_α_3 proteins is a PLC-PKC-mediated event, modulated by intracellular Ca^2+.

Na^+/H^+ exchange; protein kinases; hypertension

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_3) and D_2-like (D_2, D_3, and D_4), which are differentially expressed along the nephron (12, 33). The autocrine/paracrine function of dopamine, manifested by tubular rather than by hemodynamic mechanisms, becomes most evident during extracellular fluid volume expansion (25). This renal autocrine/paracrine function is lost in essential hypertension and in some animal models of genetic hypertension (4, 22–24, 28, 45, 56). Furthermore, disruption of the D_1 or D_3 receptor produces hypertension in mice (1, 5). In some humans with essential hypertension, renal dopamine production in response to sodium loading is impaired and may contribute to the hypertension (48). However, urinary dopamine is higher in young adults with hypertension than normotensive controls, indicating abnormality at the receptor or postreceptor levels (44). The molecular basis for the dopaminergic dysfunction in hypertension is not known but may involve an abnormal posttranslational modification of the dopamine receptor. There may be a primary defect in D_1-like receptors and an altered signaling system in the proximal tubules and thick ascending limbs of Henle that lead to reduced dopamine-mediated effects on renal sodium excretion in hypertension (25, 45).

There are currently eight cloned mammalian Na^+/H^+ exchangers (NHEs), which differ from each other in tissue distribution, sensitivity to NHE inhibitors, localization, and function (19, 21, 39). All isoforms are expressed in renal tissues, with the exception of NHE5, whereas NHE type 3 (NHE3) predominates in the apical membrane of rat renal proximal tubules (7, 27) and is largely responsible for sodium and hydrogen ion transport in this nephron segment. NHE3 activity and membrane recycling are acutely regulated by phosphorylation/dephosphorylation processes that involve protein kinase A (PKA) and/or protein kinase C (PKC) (3, 29, 37, 52, 53, 57, 58). NHE3 can also be regulated by G proteins independently of cytosolic second messengers (2, 9, 11). We recently reported that D_1 agonists decrease NHE3 activity by classic stimulation of adenyl cyclase (AC) and PKA via G_1_α proteins in rat kidney cells (42); by contrast, the D_3-mediated inhibition of NHE3 in opossum kidney cells involves both the AC-PKA and PLC-PKC pathways (15).

In the spontaneously hypertensive rat (SHR), despite normal renal production of dopamine and receptor density, there is defective transduction of the D_3 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) (13, 25, 28, 41). A consequence of the defective dopamine receptor/PKA/PKC coupling in the SHR is a decreased ability of D_1-like receptor agonists to inhibit NHE3 activity (1, 22, 25, 55). However, we have recently reported that stimulation of dopamine D_3 receptors inhibits NHE3 activity in immortalized and freshly isolated renal tubules from the SHR (43). In line with this view is the recent showing that...
D1 dopamine receptor activation increased the glomerular filtration rate (GFR), urinary volume, and sodium excretion in adult SHR and Wistar-Kyoto rats (34). This is of particular relevance in the SHR, where a defective D1 receptor signal transduction has been well characterized (23, 25, 26) and may correspond to an attempt to overcome the deficient dopamine-mediated natriuresis in genetic hypertension. The present study was carried out to evaluate the signaling events of dopamine D1 receptor-mediated inhibition of NHE activity in immortalized renal proximal tubular cells from the SHR.

METHODS

Cell culture. Immortalized renal proximal tubular epithelial cells from 4- to 8-wk-old SHR animals (54) were maintained in a humidified atmosphere of 5% CO2-95% air at 37°C. SHR cells were grown in Dulbecco’s modified Eagle’s medium nutrient mixture F-12 Ham’s (Sigma, St. Louis, MO) 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 HEPES (Sigma). For subculturing, the cells were dissociated with 0.1% trypsin-EDTA, split 1:4, and subcultured in Costar plates with 21-cm2 growth areas (Costar, Badhoevedorp, The Netherlands). For measurements of intracellular pH (pHi), PLC activity, and intracellular calcium, cells were grown in 96-well plates (Costar) or in polycarbonate filter supports (internal diameter 12 mm, Transwell, Costar), 6-well plates (Costar) and in coverslips, respectively. The cell medium was changed every 2 days, and the cells reached confluence after 3–5 days of incubation. For 24 h before 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 cm2 contained ~50 μg of cell protein.

Na+/H+ exchanger activity. Na+/H+ exchange activity was assayed as the initial rate of pHi recovery (5 min) after an acid load imposed by 20 mM NH4Cl (5 min), followed by removal of Na+ (5 min) from the Krebs’ modified buffer solution (in mM: 140 NaCl, 5.4 KCl, 1.2 CaCl2, 1.2 MgSO4, 0.3 NaH2PO4, 0.3 KH2PO4, 10 HEPES, 5 glucose, pH 7.4, adjusted with Tris base), in the absence of CO2/HCO3− (17). 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 pHi recovery periods. Intracellular pH measurements were performed in SHR cells cultured in polycarbonate filter supports or in 96-well plates (18). 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 pHi recovery period. Cells were placed in the sample compartment of a dual-scanning microplate spectrofluorometer (Spectramax Gemini, Molecular Devices, Sunnyvale, CA), 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 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 (17).

Downregulation studies. Downregulation of classical and novel PKCs was performed by overnight exposure to phorbol-12,13-dibutyrate (PDBu; 100 nM), respectively, as previously described (15, 16, 50).

PLC activity. PLC activity was assayed as previously described (16). Cells grown in six-well culture clusters were incubated for 25 min at 37°C with test compounds in Hanks’ medium. Washing the cells with ice-cold Hanks’ medium three times terminated the reaction. 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. The lysate was assayed for PLC activity using the Amplex Red phosphatidylincholine-specific PLC assay kit (Molecular Probes, Eugene, OR) and a Spectramax Gemini dual-scanning fluorescence microplate reader (Molecular Devices). In brief, PLC was monitored indirectly using 10-acetylated-3, 7-dihydroxyphenazine (Amplex Red reagent), a sensitive fluorogenic probe for H2O2. Assays were performed in 96-well plates, with 200-μl reaction volume. First, PLC converts the phosphatidylincholine (lecithin) substrate to form phosphocholine and diacrylglycerol. Then, alkaline phosphatase hydrolyses phosphocholine and choline is oxidized by choline oxidase to betaine and H2O2. Finally, H2O2 in the presence of horseradish peroxidase reacts with Amplex Red reagent in a 1:1 stoichiometry to generate the highly fluorescent product resorufin.

Intracellular calcium measurement. Intracellular calcium was measured as previously described (16). At day 4 after seeding, the glass coverslips were incubated at 37°C for 60 min with 10 μM calcium-dependent fluorescent indicator fura 2. Coverslips were then washed twice with prewarmed dye-free modified Krebs buffer [buffer composition (in mM): 114 NaCl, 1.2 CaCl2, 1.2 MgSO4, 0.3 NaH2PO4, 0.3 KH2PO4, 10 HEPES, 5 glucose, pH 7.4 adjusted with Tris base], before initiation of the fluorescence recordings. Cells were mounted diagonally in 1 × 1-cm acrylic fluorescence cuvettes, which were placed in the sample compartment of a FluoroMax-2 spectrofluorometer (Jobin Yvon-SPEX, Edison, NJ). The cuvette volume of 3.0 ml was constantly stirred and perfused at 5.0 ml/min with modified Krebs buffer prewarmed to 37°C. Under these conditions, the cuvette medium was replaced with ~150 s. After 5 min, fluorescence was measured every 5 s alternating between 340- and 380-nm excitation (2-nm slit size) at 510-nm emission (5-nm slit size). The ratio of intracellular fura 2 fluorescence at 340 and 380 nm was an index of intracellular calcium. The free Ca2+ concentration ([Ca2+]free) was calculated using the equation (20)

\[
[Ca^{2+}]_{\text{free}} = K_d \frac{(R_{\text{max}} - R_{\text{min}})}{(R_{\text{max}} - R)} [F]_{380} \frac{[F]_{380} - [F]_{380}}{[F]_{380} - [F]_{380}}
\]

where \(K_d\) is the dissociation constant, \(R\) is the ratio of each 340 nm/380 nm, \(R_{\text{max}}\) and \(R_{\text{min}}\) are the ratios at saturating and zero Ca2+ concentrations, respectively, and \(F_{380, \text{max}}\) and \(F_{380, \text{min}}\) are the maximal and minimal fluorescence intensities at 380 nm at saturating and zero Ca2+ concentration, respectively.

Drugs. Chelerythrine chloride, cholera toxin, dibutyryl cAMP, dopamine hydrochloride, forskolin, guanosine 5′-O-(3-thiotriphosphate) (GTPγS), H-89, PD-98059, pertussis toxin, phorbol-12,13-dibutyrate (PDBu), SB-203580, and U-73122 were purchased from Sigma. R-(−)-7-Hydroxy-DPAT (7-OH-DPAT), SKF-83566 hydrochloride, (±)-SKF-38393 hydrochloride, and (-)-sulpiride were obtained from Research Biochemicals International. Acetoxymethyl ester of 2′,7′-bis(carboxyethyl)-5(6)-carboxyfluorescein (BCECF-AM) fura 2 and nigericin were obtained from Molecular Probes; S-3226 3-(54)-N-isopropyldiene-2-methyl-acrylamide dihydrochloride was kindly provided by Dr. H. J. Lang (Aventis Pharma Deutschland, Frankfurt am Main, Germany).

Data analysis. Arithmetic means are given with SE or geometric means with 95% confidence values. Statistical analysis was done with a one-way ANOVA followed by the Newman-Keuls test for multiple comparisons. A P value <0.05 was assumed to denote a significant difference.

RESULTS

NHE activity was assayed as the initial rate of the Na+-dependent pHi recovery, measured after an acid load imposed by 20 mM NH4Cl, followed by removal of Na+ from the Krebs modified buffer solution, in the absence of CO2/HCO3−. In some
experiments, SHR cells were cultured in permeable polycarbonate filters to access the apical NHE activity only. In this type of assay, the pH$_i$ recovery was measured in the absence of Na$^+$ in the basal cell side. NHE activity in SHR cells was decreased by the selective dopamine D$_3$ receptor agonist 7-OH-DPAT (100 μM) in cells cultured in polycarbonate filters or in plastic clusters (Fig. 1 and Table 1). Furthermore, the basal activity of NHE and the inhibition mediated by 7-OH-DPAT (100 μM) in cells cultured in polycarbonate filters (basal 0.00893 ± 0.00067 pH U/s, n = 5; 7-OH-DPAT 0.00573 ± 0.00037 pH U/s, n = 5) were similar to that obtained in cells cultured in plastic dishes (basal 0.00942 ± 0.00033 pH U/s, n = 14; 7-OH-DPAT 0.00654 ± 0.00033 pH U/s, n = 13). The apical NHE activity was also reduced by the selective NHE3 inhibitor S-3226 (1 μM; 0.00697 ± 0.00033 pH U/s), a selective inhibitor of NHE3 (47). As shown in Fig. 1B, the effect of 7-OH-DPAT on apical NHE activity was abolished by the presence of S-3226. This strongly suggests that the NHE3 is the major isoform involved in the response to 7-OH-DPAT. As shown in Table 1, in contrast to 7-OH-DPAT that attenuated the Na$^+$-dependent pH$_i$ recovery in cells plated in plastic dishes, the selective D$_3$-like receptor agonist SKF-38393 was devoid of any effect. The D$_2$-like receptor antagonist S-sulpiride blocked the inhibitory effect of 7-OH-DPAT on NHE activity. Altogether, these results show that NHE activity in immortalized proximal tubular SHR cells can be reduced by dopamine D$_3$ receptor but not D$_2$-like receptor stimulation.

Next, we evaluated the effects of GTP$\gamma$S, a nonhydrolyzable GTP analog, to confirm the involvement of G proteins in the regulation of NHE3. Treatment of SHR cells with increasing concentrations of GTP$\gamma$S (Table 1) reduced NHE3 activity, in a concentration-dependent manner. Cholera toxin (CTX) and pertussis toxin (PTX) ribosylate the $\alpha$-subunit of the G$_s$ and G$_i/o$ classes of G proteins, respectively. The effect of 7-OH-DPAT was abolished by overnight treatment of SHR cells with PTX (100 ng/ml), but not with CTX (500 ng/ml) (Table 1). These results suggest that dopamine D$_3$ receptors stimulated by 7-OH-DPAT in SHR cells are coupled to PTX-sensitive G proteins of the G$_i/o$ class. To further elucidate the coupling of dopamine D$_3$ receptors to G proteins, additional studies were performed in cells treated overnight with antibodies raised against rat G$_{\alpha}$, G$_{q/11}$, G$_{\alpha}$3, G$_{\alpha}$1.2, or G$_{\beta}$ proteins. Antibodies were complexed with liposomes to facilitate delivery into the cytosol (15, 16). As shown in Fig. 2, the inhibitory effect of 7-OH-DPAT on NHE3 was abolished in cells treated with the anti-G$_{\alpha}$ antibody, but not in cells treated with the anti-G$_{\alpha}$, anti-G$_{q/11}$, anti-G$_{\alpha}$1.2 or anti-G$_{\beta}$ antibodies.

NHE3 activity is acutely regulated by phosphorylation/dephosphorylation, a process that may involve PKA and/or PKC and membrane recycling in intact cells (3, 29, 36, 37, 52, 53, 57). To address these issues, cells were treated with selective antagonists of PKA (H-89) or PKC (chelerythrine). As shown in Fig. 3, H-89 abolished the inhibitory effects of forskolin, an adenyl cyclase agonist, on NHE3 activity, but not that of 7-OH-DPAT. These suggest that PKA did not participate in the signal transduction pathway of dopamine D$_3$ receptor activation. To evaluate the contribution of PKC in signal transduction pathway coupled to the dopamine D$_3$ receptor, the effect of PDBu, an activator of classical and novel PKCs, was examined. Treatment of SHR cells with increasing

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**Table 1. NHE activity (% of control) in SHR cells in the absence and presence of 7-OH-DPAT (100 μM), SKF 38393 (1 μM), S-sulpiride (1 μM), GTP$\gamma$S (0.1, 0.3, and 1 mM), CTX (500 ng/ml), or PTX (100 ng/ml)**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>NHE Activity (%) of Control</th>
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<tbody>
<tr>
<td>Vehicle</td>
<td>100±4 (n=23)</td>
</tr>
<tr>
<td>7-OH-DPAT</td>
<td>58±3 (n=13)*</td>
</tr>
<tr>
<td>SKF-38393</td>
<td>91±4 (n=15)</td>
</tr>
<tr>
<td>S-sulpiride</td>
<td>100±5 (n=14)</td>
</tr>
<tr>
<td>S-sulpiride + 7-OH-DPAT</td>
<td>96±7 (n=8)</td>
</tr>
<tr>
<td>GTP$\gamma$S 0.1 mM</td>
<td>84±4 (n=8)*</td>
</tr>
<tr>
<td>GTP$\gamma$S 0.3 mM</td>
<td>70±4 (n=8)*</td>
</tr>
<tr>
<td>GTP$\gamma$S 1 mM</td>
<td>44±2 (n=2)*</td>
</tr>
<tr>
<td>CTX</td>
<td>100±4 (n=8)</td>
</tr>
<tr>
<td>CTX + 7-OH-DPAT</td>
<td>70±4 (n=8)*†</td>
</tr>
<tr>
<td>PTX</td>
<td>100±8 (n=8)</td>
</tr>
<tr>
<td>PTX + 7-OH-DPAT</td>
<td>105±10 (n=8)</td>
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</tbody>
</table>

Values are means ± SE. Significantly different from values for vehicle (*$P < 0.05$) and corresponding 7-OH-DPAT control values (†$P < 0.05$). SHR, spontaneously hypertensive rat; CTX, cholera toxin; PTX, pertussis toxin.

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Fig. 1. A: assessment of intracellular pH (pH$_i$) in absence and presence of 7-OH-DPAT (100 μM) after an acid load imposed by exposure to NH$_4$Cl, followed by Na$^+$ removal in apical cell side, in the absence of Na$^+$ in the basal cell side, in SHR cells cultured in polycarbonate filters. B: effect of 7-OH-DPAT (100 μM) in absence or presence of S-3226 (1 μM) on apical Na/H exchanger (NHE) activity under $V_{\text{max}}$ conditions as the initial rate of Na$^+$-dependent pH$_i$ recovery, in the absence of Na$^+$ in the basal cell side, in spontaneously hypertensive rat (SHR) cells cultured in polycarbonate filters. Symbols and columns represent means of 3 or 6 experiments per group and vertical lines show SE. *Significantly different from control values ($P < 0.05$).
concentrations of PDBu (10–3000 nM; Fig. 4A) reduced the NHE3 activity. As shown in Fig. 4B, chelerythrine (1 μM) antagonized the effects of both PDBu (100 nM) and 7-OH-DPAT (100 μM). To confirm the involvement of PKC on the inhibition of NHE3 evoked by dopamine D1 receptor stimulation, complementary studies involving downregulation of PKC were performed. To promote classic and novel PKC downregulation, SHR cells were incubated overnight in the presence of PDBu (100 nM). Under these conditions, PDBu or 7-OH-DPAT was devoid of effect on NHE3 activity (Fig. 4C). Several studies, including some of our group, demonstrated a critical role of PLC in modulation of Na\(^{+}\)-K\(^{+}\)-ATPase and NHE3 activity evoked by dopamine receptor stimulation (14, 16, 26, 42, 51). To evaluate the involvement of PLC in the signal transduction pathway coupled to dopamine D3 receptor-mediated inhibition of NHE3, we tested the PLC inhibitor U-73122 (8) on the effect of 7-OH-DPAT. As shown in Fig. 5, U-73122 (3 μM) prevented the inhibitory effects of 7-OH-DPAT on NHE3 activity. As shown in Fig. 6, 7-OH-DPAT (100 μM) and PDBu (100 nM) increased PLC activity in SHR cells, whereas U-73122 (3 μM) effectively reduced the PLC activity. The role of PLA\(_2\)-arachidonic acid-20-HETE pathway has also been reported in the signaling subsequent to dopamine receptor stimulation, namely in inhibition of Na\(^{+}\)-K\(^{+}\)-ATPase in the proximal tubules (40). The result that arachidonic acid was devoid of an effect (Table 2) in NHE3 activity is compat-
Table 2. NHE3 activity (% of control) in SHR cells in the absence and presence of 7-OH-DPAT (100 μM), AA (1 μM), PD-98059 (10 μM), or SB-203580 (10 μM)

<table>
<thead>
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<th>Treatment</th>
<th>NHE Activity (%) of Control</th>
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<tbody>
<tr>
<td>Vehicle</td>
<td>100±4</td>
</tr>
<tr>
<td>7-OH-DPAT</td>
<td>74±3*</td>
</tr>
<tr>
<td>AA</td>
<td>101±5</td>
</tr>
<tr>
<td>PD 98059</td>
<td>100±4</td>
</tr>
<tr>
<td>PD 98059 + 7-OH-DPAT</td>
<td>78±2*†</td>
</tr>
<tr>
<td>SB 203580</td>
<td>100±6</td>
</tr>
<tr>
<td>SB 203580 + 7-OH-DPAT</td>
<td>79±5*†</td>
</tr>
</tbody>
</table>

Values are means ± SE of 7 or 8 experiments per group. Significantly different from values for vehicle (*P < 0.05) and corresponding 7-OH-DPAT control values (#P < 0.05). AA, arachidonic acid.
7-OH-DPAT- and the PDBu-induced decrease in NHE3 activity (Fig. 8D). To confirm the effect of A-23187 and thapsigargin on intracellular Ca\(^{2+}\), changes in Ca\(^{2+}\) intracellular concentrations were measured with a fluorescent method. In SHR cells, the intracellular free Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\)) was 33.8 ± 0.2 nmol/l (n = 5). As shown in Fig. 9, stimulation of D\(_3\) dopamine receptors by 7-OH-DPAT (100 µM) had no effect on [Ca\(^{2+}\)]\(_i\) (32.7 ± 0.1 nmol/l; n = 5), whereas both A-23187 (3 µM) and thapsigargin (3 µM) markedly raised intracellular Ca\(^{2+}\) in SHR cells. The maximal concentration of free Ca\(^{2+}\) obtained for A-23187 (3 µM) and thapsigargin (3 µM) was 162.9 ± 2.8 nmol/l (n = 4) and 79.3 ± 2.6 nmol/l (n = 3), respectively.

**DISCUSSION**

We previously reported that stimulation of dopamine D\(_3\) receptors inhibits NHE3 activity in immortalized and freshly isolated renal tubules from the SHR (43). In the present study, it is demonstrated that in immortalized proximal tubular cells from the SHR, downstream to the G\(_{i3}\)-coupled dopamine D\(_3\) receptor, NHE3 inhibition involves the PLC-PKC system. Furthermore, Ca\(^{2+}\) appears to have an important role as modulator of dopamine D\(_3\) receptor-induced and PKC-mediated decrease in NHE3 activity in SHR cells.

Several lines of evidence suggest that the inhibitory effects evoked by the dopamine D\(_3\) receptor on NHE3 activity in SHR cells are coupled to G\(_{i}\) protein. Two observations support this view. First, is the finding that inhibition of NHE3 activity by 7-OH-DPAT was abolished by overnight treatment of SHR cells with PTX, but not with CTX. In a second series of experiments, we used antibodies raised against rat G\(_{i3}\), G\(_{q/11}\), G\(_{i/1}\), G\(_{i1}\), or G\(_{q}\) proteins to block interactions of G proteins with dopamine D\(_3\) receptors. Under these experimental conditions, inhibition of NHE3 activity by 7-OH-DPAT was abolished only in cells treated with the anti-G\(_{i}\) antibody.

The involvement of PKA on the signal transduction activated by dopamine D\(_3\) receptors was also addressed. Several findings suggest the involvement of PKA in regulation of NHE3 activity (29, 36, 52). However, selective inhibition of PKA with H-89 prevented the decrease in NHE3 activity by forskolin, but not by 7-OH-DPAT. This suggests that PKA does not participate in the signal transduction pathway following dopamine D\(_3\) receptor activation, although it has a role in cAMP-dependent regulation of NHE3 activity.

Activation of PLC is considered to be one of the cellular signaling events involved in dopamine-mediated natriuresis (26, 51). In fact, the modulation of Na\(^{+–}\)K\(^{+}\) ATPase and NHE3 activity by dopamine receptor stimulation has been related to PLC activation (14, 16, 42). The finding that the PLC inhibitor U-73122 prevented the inhibitory effect of 7-OH-DPAT on NHE3 activity and that dopamine D\(_3\) receptor stimulation significantly increased PLC activity suggests that downstream dopamine D\(_3\) receptor transduction includes G\(_{i3}\)-PLC activation. Because activation of PKC is one of the major consequences of PLC signaling (6, 38), the effects of PDBu, a direct activator of PKC, and chelerythrine, a PKC inhibitor, were also evaluated. The findings reported here in SHR cells show that PDBu reduced NHE3 activity in a concentration-dependent manner, which was prevented by the PKC inhibitor chelerythrine. Similarly, PKC inhibition by chelerythrine prevented the decrease in NHE3 activity by 7-OH-DPAT. These results suggest the involvement of classic or novel PKCs in the signal transduction pathway following dopamine D\(_3\) receptor activa-

![Fig. 8: A: effect of varying concentrations of thapsigargin (0.1–10 µM) on NHE3 activity. B: effect of 7-OH-DPAT (100 µM) and PDBu (100 nM) in the absence and presence of thapsigargin (3 µM) on NHE3 activity. Columns represent means of 6–23 experiments per group and vertical lines show SE. Significantly different from control values (*P < 0.05) and values for cells treated only with 7-OH-DPAT or PDBu (#P < 0.05).](http://ajprenal.physiology.org/)

![Fig. 9: Representative tracing of the effect of A-23187 (3 µM), thapsigargin (3 µM), or 7-OH-DPAT (100 µM) when monolayers of SHR cells were treated with Krebs buffer with 0.5 of Ca\(^{2+}\). The test compounds were added at 600 s in perfusion, and duration of perfusion with test compounds is indicated by the arrow.](http://ajprenal.physiology.org/)
tion. This view was confirmed in studies in which downregu-
lation of PKC abolished the inhibitory effects of PDBu and
7-OH-DPAT on NHE3 activity. The involvement of PKC is
consistent with previous reports showing that NHE3 activity is
cutely regulated by PKC-dependent phosphorylation/dephos-
phorylation processes (3, 53). We suggest that a single se-
quence of events downstream of dopamine D3 receptor ac-
恬ation with PLC activation prior to PKC activation is involved in
the regulation of NHE3 activity in SHR cells. However, PDBu
markedly increased PLC activity, possibly as a consequence of
the potentiation of PLC signaling by PKC, as has been shown for several receptors including the D1 receptor (46, 58).

One consequence of PLC activation is an increase in in-
tracellular Ca2+. However, the role of Ca2+ in NHE3 regu-
lation is not clear, although there is evidence suggesting that Ca2+
might modulate the activity of NHE3 (31). Therefore, we
evaluated the relationships among Ca2+, PKC activation, and
inhibition of NHE3 activity during dopamine D3 receptor ac-
恬ation. The findings that A-23187 did not change NHE3 ac-
恬ity and that the decrease in NHE3 activity with thapsig-
argin was observed only at high concentrations suggest that
increases in intracellular Ca2+ may not have a direct role in
the regulation of NHE3 activity. It should be underscored, how-
ever, that A-23187 and thapsigargin, at concentrations that did
not affect NHE3 activity, completely prevented the inhibition
of NHE3 activity by 7-OH-DPAT and PDBu. These observa-
tions suggest a role of intracellular Ca2+ on a PKC-dependent
modulation of NHE3 activity. Cheng et al. (10) reported that
the regulation of Na+/K+-ATPase activity by PKC is de-
pent by the intracellular Ca2+-concentration and that its inhib-
itory effect on Na+/K+-ATPase is reversed by increases in
intracellular Ca2+. In agreement with this hypothesis, reguc-
colin, a Ca2+-binding protein, caused an inhibition of PKC
activity in rat renal cortex cytosol, only when Ca2+ is present
(30). The finding that Ca2+ modulates the regulation of NHE3 activity by PKC has an important physiological implication, i.e., PKC-mediated inhibition of NHE3 activity involving PKC
activation might occur with no or minor changes in intracel-
lular Ca2+. By contrast, when PKC activation is accompanied
by increases in intracellular Ca2+ changes in NHE3 activity do
not occur.

In conclusion, the transduction mechanisms set into motion
following the coupling of the dopamine D3 receptor to Gα3
proteins may involve PLC and PKC activation in a single
sequence of events that results in inhibition of NHE3 activity.
Intracellular Ca2+ appears to have a modulatory role in dopamine
D3 receptor- and PKC-mediated decrease in NHE3 ac-
tivity in SHR cells.

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DOPAMINE-MEDIATED NHE3 INHIBITION IN SHR


