Regulation of NHE3 activity by G protein subunits in renal brush-border membranes

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The sodium/hydrogen exchanger (NHE) is the major transporter of sodium across the luminal membrane of the proximal tubule (1). Six NHE isoforms are found in mammals, and all are expressed in renal tissue, with the exception of the NHE5 isoform, which is found in the central nervous system, testes, spleen, and skeletal muscle (1). The NHE3 isoform is the only NHE isoform expressed in rat renal brush-border membranes (BBM). Therefore, it is responsible for the amiloride-sensitive Na+ transport in BBM vesicles (BBMV) (2–4, 6, 7, 22, 41). NHE3 activity is regulated by phosphorylation/depolymerization processes and membrane recycling in intact cells (14, 23, 42, 43). NHE3 can also be regulated by G proteins independent of cytoplasmic components and second messengers; however, the G protein subunits involved in this regulation are not known. Therefore, we studied G protein subunit regulation of NHE3 activity in renal brush-border membranous vesicles (BBMV) in a system devoid of cytoplasmic components and second messengers. Basal NHE3 activity was not regulated by Gs or Gi, because antibodies to these G proteins by themselves were without effect. The inhibitory effect of D1-like agonists on NHE3 activity was mediated, in part, by Gi, because it was partially reversed by anti-Gi antibodies. Moreover, the amount of Gs that coimmunoprecipitated with NHE3 was increased by fenoldopam in both brush-border membranes and renal proximal tubule cells. Furthermore, guanosine 5′-O-(3-thiotriphosphate) but not guanosine 5′-O-(2-thiotriphosphate), the inactive analog of GDP, increased the amount of Gs that coimmunoprecipitated with NHE3. The α2-adrenergic agonist, UK-14304 or pertussis toxin (PTX) alone had no effect on NHE3 activity, but UK-14304 and PTX treatment attenuated the D1-like receptor-mediated NHE3 inhibition. The ability of UK-14304 to attenuate the D1-like agonist effect was not due to Gi, because the attenuation was not blocked by anti-Gi antibodies or by PTX. Anti-Gr(α) antibodies, by themselves, slightly inhibited NHE3 activity but had little effect on D1-like receptor-mediated NHE3 inhibition. However, anti-Gr(α) antibodies reversed the effects of UK-14304 and PTX on D1-like agonist-mediated NHE3 inhibition. These studies provide concrete evidence of a direct regulatory role for Gi, independent of second messengers, in the D1-like-mediated inhibition of NHE3 activity in rat renal BBMV. In addition, βγ dimers of heterotrimeric G proteins appear to have a stimulatory effect on NHE3 activity in BBMV.

sodium/hydrogen exchanger isoforms; proximal tubule; kidney

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These heterotrimeric G proteins are composed of Ga, Gb, and Gc subunits, where the Ga subunits bind and hydrolyze GTP, whereas the Gb and Gc subunits exist as a tightly bound dimer. The Ga subunit and Gb/Gc dimer directly regulate effector proteins, including enzymes and ion channels (25, 28). Some effectors, e.g., adenyl cyclase, are regulated by both the G protein Ga subunits and Gb/Gc dimers (37). G protein subunits may also regulate NHE3 activity in renal BBM independent of second messengers (5, 10, 32); however, the G protein subunit mediating this regulation is not known. Therefore, a secondary aim of this study is to determine the role of the G protein subunits Ga, Gb, and Gc in the second messenger-independent regulation of NHE3 activity in renal BBM by D1-like receptors.

**METHODS**

Preparation of renal BBM. Male Wistar-Kyoto (WKY) rats (Taconic, Germantown, NY), 9–16 wk old, fed on regular Purina rat chow diet were anesthetized with an intraperitoneal injection of pentobarbital sodium (50 mg/kg body wt), and the kidneys were harvested. We wanted to measure the activity of NHE3, the NHE isoform expressed in BBMV (2, 4, 41), and the kidneys were harvested. We wanted to measure the activity of NHE3, the NHE isoform expressed in BBMV (2, 4, 41). BBMV were isolated from the outer two-thirds of the cortex, which was separated from the medulla to avoid inclusion of medullary rays and the medullary thick ascending limb of Henle. These maneuvers ensured isolation of nephrons that do not express NHE1 (3, 4, 6, 7, 22). The presence of NHE1 in basolateral membranes of deeper nephrons could contaminate the BBM preparation and thus confound interpretation of our results (22). These BBMs were devoid of NHE1 protein, as determined by immunoblotting (data not shown), in agreement with other reports (3, 22).

BBMs were isolated by MnCl2 precipitation and differential centrifugation, as previously reported (10, 12, 31). The BBMs were suspended in (mM) 150 KCl with 25 2-(N-morpholino)ethanesulfonic acid (MES) and adjusted to pH 5.5 with KOH (“inside” buffer). The BBMs spontaneously form vesicles while incubating on ice for 60 min. Subsequently, depending on the experiment, drugs, or their vehicle, and antibodies, or other use for cell control (heat-denatured antibodies to assure the same sodium and protein concentrations among groups), were added to the BBMs.

Measurement of NHE3 activity. NHE3 activity was assayed by measuring the 3-s 100 µM 5-((R)-methyl-2-morpholino)-amiloride (MIA)-sensitive 22Na	extsuperscript{+} uptake (difference between 22Na	extsuperscript{+} uptake in the absence of amiloride or its analogs and 22Na	extsuperscript{+} uptake in presence of amiloride or its analogs) as described (10–12, 21, 30–32, 39). The validity and reliability of this assay have been well established in many laboratories, including our own (5, 10–12, 27, 32, 39). In agreement with Wu et al. (41), NHE activity in BBMV was due to NHE3 isoform. NHE activity in our BBMV preparations was resistant to 5-(N-ethyl-N-isopropyl)-amiloride (EIPA); NHE1 is 100 times more sensitive to the inhibitory effect of EIPA, relative to its effect on the NHE3 isoform (19, 30). Uptake of 22Na	extsuperscript{+} into BBMV was measured at 24°C by using the Millipore rapid filtration technique with 0.65-µm nitrocellulose filters (10–12). The BBMV (after vesicle formation) were incubated with receptor agonists/drugs for 30 min before 22Na	extsuperscript{+} uptake. When antibodies were used, they were added to BBM before vesicle formation (i.e., 90 min before 22Na	extsuperscript{+} uptake). When drugs were used with the antibodies, the BBM were exposed to the antibodies 60 min before vesicle formation, and the receptor agonists/drugs were added after vesicle formation 30 min before 22Na	extsuperscript{+} uptake, as stated previously. 22Na	extsuperscript{+} uptake was then determined by mixing 20 µl of the membrane vesicle suspension (150–350 µg protein) and 30 µl of uptake medium and incubating for 3 s at 24°C. The final concentration was (in mM) of 142 KCl, 14.7 KOH, 10 MES, 9 HEPES, and 1 NaCl (containing 0.1 to 0.2 µCi of 22Na), pH 7.5. Three seconds after mixing, transport was halted by adding 2 ml of ice-cold stop buffer (in mM) 150 KCl, 15 HEPES, 0.1 MIA, pH 7.5. The studies were performed in the presence of an outwardly directed pH gradient (pHin = 5.5, pHout = 7.5) and an inwardly directed Na	extsuperscript{+} gradient ([Na]out = 1 mM, [Na]in = 0 mM). Drug or antibodies that required access to the interior of the vesicles were added to the membrane suspensions before vesicle formation, as described above and previously reported (6, 11, 12, 39).

Pertussis toxin treatment of BBM. After the isolation of BBM, the membranes were suspended in an incubation buffer containing (in mM) 100 Tris(hydroxymethyl)-aminomethane hydrochloride, 40 dithiothreitol, 20 thymidine, 5 ethylenediaminetetraacetic acid, 1 MgCl2, 1 adenosine-5'-triphosphate, and 1 ß-nicotinamide adenine dinucleotide (NAD), pH 7.4. The suspension was centrifuged at 18,000 rpm for 20 min and then resuspended in the above buffer. Pertussis toxin (PTX) was preincubated in incubation buffer for 10 min at 30°C to activate PTX (16); buffers without PTX were treated similarly as controls. Subsequently, the BBMs were incubated in incubation buffer with or without PTX (25 µg PTX/mg protein) at 30°C for 1 h to ADP ribosylate Ga (16). The incubation buffer- and PTX-treated membranes were centrifuged at 18,000 rpm for 20 min. The pellet was washed and resuspended in a volume of inside buffer (equal to 100 times the pellet volume) and centrifuged at 18,000 rpm for 20 min (2×). The resulting pellet was resuspended with inside buffer to an approximate protein concentration of 1 mg/ml. Protein concentrations were determined by the Lowry method. The BBMV were then used for transport studies as described in the preceding section.

ADP ribosylation studies. The BBMs were treated in a manner similar to that described for PTX, except that 32P-labeled NAD was used (16). The BBMs were solubilized in Laemmli Tris-glycine and SDS-PAGE denaturing, reducing buffer (catalog #LC2675, Novex, Diag. CA) and boiled for 3 min. The suspensions of solubilized BBMV with and without PTX were loaded on a 12% Tris-glycine gel and electrophoresed at 100 V for 120 min.

Immunoprecipitation studies. Three types of experiments were performed. In the first set of experiments, BBM were treated with GTP(S) (10-3 M) or an equal volume of incubation buffer containing (in mM) 150 NaCl, 10 MgCl2, and 2 Tris·HCl, pH 7.5 for 30 min at room temperature and then centrifuged for 20 min at 18,000 rpm, 4°C. In the second set of experiments, plasma membranes or immortalized renal proximal tubule cells from WKY rats (40) were incubated with vehicle, GTP(S) (10-3M), or guanosine 5'-O-(2-thiodiphosphate) (GDPβS; 10-4M) for 30 min at 37°C. In the third set of experiments, immortalized renal proximal tubule cells were incubated with vehicle or fenoldopam (5 x 10-6 M) for 10 min at 37°C. The cells were lysed with ice-cold lysis buffer [PBS with 1% Nonidet P-40, 0.1% SDS, 0.5% deoxycholate, 1 mM phenylmethylsulfonyl fluoride, 10 µg/ml aprotinin, and 10 µg/ml leupeptin (1 mM sodium vanadate was added in the immortalized proximal tubule studies)] and centrifuged at 15,000 rpm for 20 min. The lysates were then incubated with 4 µl of antibody against the protein of interest or 4 µl of normal rabbit IgG (as a control) for 1 h at 4°C. Thereafter, 20 µl of 25% protein A agarose (Santa Cruz Biotech, Santa Cruz, CA) were added and incubated overnight on a rocking plat-
form at 4°C. The immunoprecipitates were pelleted, washed with lysis buffer, suspended in sample buffer, and boiled for 10 min. In some studies using anti-NHE3 antibody for immunoprecipitation and anti-Gsα for immunoblotting, 2-mercaptoethanol (100 mM) was used instead of β-mercaptoethanol. In this experimental setup, the samples were incubated for 120 min at 24°C to cleave GG into 75-kDa fragments. This allowed better visualization of proteins with molecular sizes of ~50 kDa, e.g., Gsα; similar results were obtained using 150 mM 2-mercaptoethanol incubated for 30 min at 37°C.

Western blotting. The proteins were separated by electrophoresis on 7.5% SDS-PAGE and then electrophoretically transferred onto nitrocellulose membranes. The transblots were probed with the indicated antibodies and detected by hydrogen peroxidase-labeled secondary antibody and chemiluminescence detection reagents. Quantification of the immunoblots was performed; the density of the area of each immunoblot was quantified using Quantiscan (Biosoft, Ferguson, MO).

Statistical analysis. Data are expressed as means ± SE. Differences within groups were analyzed by analysis of variance (ANOVA) repeated measures, followed by Scheffe’s test; paired t-test was used when only two groups were compared. Differences among groups were analyzed by one-way analysis of variance, followed by Scheffe’s test; if only two groups were compared, unpaired t-test was used.

Drugs. The chemicals used were MIA, EIPA, SKF-81297, and UK-14304 (Research Biochemicals, Natick, MA); 5,8,11,14-eicosatetraynoic acid (ETYA; Biomol Research Laboratories, Plymouth Meeting, PA); PTX, GTPγS, and Gsα standard (Calbiochem, La Jolla, CA); 2-mercaptoethanol- HCl (Pierce, Rockford, IL). All other reagents were bought from Sigma (St. Louis, MO). Anti-NHE1 monoclonal antibodies were purchased from Chemicon, Temecula, CA. Anti-NHE3 antibodies were raised against synthetic oligopeptides from the amino acid sequence of rat NHE3 (amino acids 633–646; Research Genetics, Huntsville, AL) (2).

RESULTS

NHE3 activity in BBMV. The NHE isoform responsible for a particular NHE activity can be determined by its sensitivity to amiloride analogs (30). NHE3 is relatively insensitive to EIPA, whereby the EIPA-sensitive isotypes are fully inhibited by 5 × 10⁻⁷ M EIPA (30). NHE3 is the only known NHE isoform found in the BBM of renal proximal tubules that is relatively insensitive to EIPA (2–4, 6, 7, 22, 41). Therefore, we determined the effect of varying concentrations of EIPA (10⁻⁹–10⁻⁵ M) on ²²Na⁺ uptake into BBMV. ²²Na⁺ uptake into BBMV was not affected by 10⁻⁶ M EIPA, indicating that all of the measured NHE activity in BBM was due to NHE3 and not to NHE1 (30) or other EIPA-sensitive NHE isoforms. ²²Na⁺ uptake at 1–2 h was assumed to represent equilibrium values and also served as an index of vesicle size (21). In the current and previous reports, no differences among drug/antibody or vehicle-treated membranes at 1–2 h were noted, indicating that vesicle sizes were similar among the groups.

Basal NHE3 activity in BBMV was 1.95 ± 0.16 nmol ²²Na⁺·mg protein⁻¹·min⁻¹. Pretreatment of BBM with dopamine or D₁-like agonists inhibited the amiloride-sensitive Na⁺ transport (3-s uptake) up to 70% in a dose- and time-dependent manner (Fig. 1A, Refs. 10, 12). In contrast, receptor ligands had no effect on EIPA or MIA-insensitive Na⁺ transport. Previous experiments had established that dopaminergic inhibition of amiloride-sensitive Na⁺ transport cannot be explained by an increase of the amiloride-sensitive Na⁺ transport rate, collapse of the proton gradient driving Na⁺/H⁺ exchange, or a decrease in vesicle size (9, 12). The amiloride-sensitive Na⁺ transport includes all contributions of Na⁺ movement through Na⁺/cotransporters, such as Na⁺/glucose transporter (SGLT)-1, SGLT-2, sodium-amino acid cotransporters, and sodium-phosphate cotransporters. The amiloride-sensitive Na⁺ transport was unchanged by pretreatment with dopamine or D₁-like agonist under the experimental conditions of Figs. 1A and B, 2, 3, 4, A-C (absence of phosphate, glucose, or amino acids in the incubation buffer). Therefore, the decrease in the amiloride-sensitive Na⁺ transport rate reflects inhibition of NHE3.

**Fig. 1.** A: best-fit analysis of dose response inhibition by dopamine and D₁-like receptor agonists, SKF-81297 and fenoldopam, on sodium-hydrogen exchanger (NHE) 3 activity in brush-border membrane vesicles (BBMV) isolated from rat renal cortex (n = 3–7/drug concentration). NHE3 activity was significantly inhibited by all concentrations, except for fenoldopam at 5 × 10⁻⁷ M. B: inhibitory effect of SKF-81297 (5 × 10⁻⁶ M) on NHE3 activity in BBMV incubated with and without antibodies for the Gsα subunit (1:100 dilution). *P < 0.05 vs. others, †P < 0.05 vs. D₁-like agonist, ANOVA, Scheffé’s test.
This inhibition in BBM is seen in the absence of added ATP and GTP.

\( \text{Gs}_\alpha \) mediates D\(_1\)-like agonist inhibition of NHE3 activity in BBMV. Although, NHE3 activity is regulated by phosphorylation/dephosphorylation processes and membrane recycling (14, 23, 42, 43), it can be regulated also by G proteins, independent of cytoplasmic second messengers (5, 10). In agreement with previous studies, dopamine and two different D\(_1\)-like agonists, SKF-81297 and fenoldopam, decreased NHE3 activity in BBMV (Fig. 1A), presumably independent of cAMP (10, 11). Other investigators have shown that dopamine does not stimulate cAMP production in BBMV unless ATP is added (32). Moreover, inhibition of adenylyl cyclase, PKA, and PKC activities did not prevent the inhibitory action of fenoldopam on NHE3 activity in rat BBMV (10). Because dopamine can stimulate phospholipaseA\(_2\) activity in BBM (32), we also studied the effect of ETYA on NHE3 activity at a concentration (10\(^{-4}\) M)
known to inhibit phospholipase A₂, lipoxigenase, and monooxygenase activities (38). In our study, the inhibitory effect of SKF-81297 was not affected by ETYA (data not shown). These results demonstrate that the inhibitory effect of D₁-like agonists on NHE3 activity in BBMV was not caused by PKA or PKC activation or by eicosanoids.

GTPγS incorporated inside BBMVs inhibited NHE3 activity (5, 10). Thus NHE3 activity can be inhibited by G proteins independent of cytoplasmic second messengers (5, 10). In the current study, we confirm the ability of GTPγS (10⁻³ M) to decrease NHE3 activity (control amiloride-sensitive ²²Na⁺ uptake = 2.99 ± 0.24 vs. 2.06 ± 0.16 nmol Na⁺·mg protein⁻¹·min⁻¹ with GTPγS, P < 0.05, paired t-test). In contrast, GTPγS added to the "outside" of BBMV did not significantly affect NHE3 activity (2.52 ± 0.24 nmol ²²Na⁺·mg protein⁻¹·min⁻¹, P > 0.05, t-test) (9).

The inhibition of NHE3 activity by the D₁-like agonist SKF-81297 was caused in part by Gₛα, because antibodies directed against this G protein subunit (1:100 dilution) partially reversed the D₁-like inhibition in BBMV (SKF-81297 + heat-denatured anti-Gₛα antibody = 59 ± 5%, n = 12, SKF-81295 + anti-Gₛα antibody = 36 ± 4%, n = 8) of NHE3 activity by ~39 ± 5%; anti-Gₛα antibody by itself (control) was without effect (Fig. 1B). Heat-denatured anti-Gₛα antibody had a minimal effect on NHE3 activity when compared with vehicle treatment alone (data not shown). All groups were compared with a vehicle control, as well as a control consisting of heat-denatured anti-Gₛα antibodies in the place of active antibodies, to ensure ion and protein concentrations are the same between groups. The anti-Gₛα antibody used in this study has been shown to be specific (33). In the current report, the anti-Gₛα antibody recognized a recombinant 45-kDa Gₛα standard (not shown) as well as 45- and 52-kDa proteins in immunoblots of BBM (see, for example, Fig. 6A). The ability of anti-Gₛα antibody to partially attenuate the inhibitory effect of SKF-81297 on NHE3 activity was caused by anti-Gₛα antibody, because heat-denatured anti-Gₛα antibody (control studies) had no effect on D₁-like inhibition of NHE3 activity (Fig. 1B). The inability of anti-Gₛα antibody to completely block the inhibitory action of SKF-81297 may be related to the fact that only a limited amount of antibody (1:100) can be "loaded" inside the vesicle; 1,000-fold dilution of anti-Gₛα antibody had no effect, whereas 1:10 dilution resulted in variable uptake of ²²Na⁺ (data not shown).

Gₛα does not mediate the ability of an α₂-adrenergic agonist to reverse the D₁-like inhibition of NHE3 activity in BBMV. Norepinephrine and angiotensin II oppose D₁-like agonist inhibition of NHE3 activity in the luminal membrane of renal proximal tubule cells caused, in part, by inhibition of adenyl cyclase activity (8). UK-14304 by itself had no effect (Fig. 2), probably because NHE3 activity must be inhibited first to demonstrate a stimulatory effect (8). However, in BBMV where cAMP cannot be generated, UK-14304, an α₂-adrenergic agonist, was able to partially reverse (22–55%) the D₁-like agonist inhibition of NHE3 activity (Fig. 3). The Gₛα subunit did not appear to be involved in the current experimental setup, because anti-Gₛα, antibodies (1:100 dilution) did not affect the ability of UK-14304 to counteract the D₁-like agonist inhibition of NHE3 activity (Fig. 3). Gₛα subunit is the predominant Gₛα isoform expressed in renal proximal tubules (35). The anti-Gₛα antibody used in this study has been shown to be specific (34). In the current report, the anti-Gₛα antibody recognized a 41-kDa protein that corresponded with the protein ADP³²P ribosylated by PTX in BBMV (data not shown). Lower dilutions of anti-Gₛα antibody (1:10 dilution), as with the lower dilutions of anti-Gₛα antibody, resulted in variable uptakes of ²²Na (data not shown). In addition, anti-Gₛα antibodies did not influence basal or D₁-like agonist inhibition of NHE3 activity (Fig. 3). All groups were compared with a vehicle control as well as a vehicle plus heat-denatured anti-Gₛα (1:100 dilution) antibody control to ensure that concentrations of ions and proteins are the same between groups.

The inability of anti-Gₛα antibodies to affect NHE3 activity may have been due to the presence of other Gₛα subunit isoforms. Therefore, to prove that Gₛα subunits did not mediate the α₂-adrenergic agonist attenuation of D₁-like agonist inhibition of NHE3 activity, we performed additional studies in BBMV treated with PTX, a toxin known to inactivate Gₛα through ADP ribosylation (16). Incubation of BBMV with PTX at a concentration that ADP ribosylated a 41-kDa protein had no effect on basal NHE3 activity compared with the vehicle (control) or α₂-adrenergic agonist [UK-14304 (5 × 10⁻¹⁰ M)]-treated group. The vehicle control groups were studied concurrently and consisted of BBMVs incubated in the same buffers without PTX. PTX treatment did not affect basal NHE3 activity; it also did not alter any UK-14304 effect. Thus, in our studies, Gₛα did not influence NHE3 activity. However, after PTX treatment, there was no longer a difference in NHE3 activity between D₁-like receptor agonist treatment alone and D₁-like receptor agonist plus α₂-adrenergic receptor treatment (Fig. 2). This was not caused by a reduction in the stimulatory effect of the α₂-adrenergic agonist on NHE3 activity but, rather, by a diminution (43% ± 5%) in the inhibitory effect of the D₁-like agonist [SKF-81297 (5 × 10⁻⁶ M)].

β/γ dimer mediates the ability of α₂-adrenergic agonist to reverse the D₁-like inhibition of NHE3 activity in BBMV. Because Gₛα did not mediate the "stimulatory" effect of α₂-adrenergic receptor activation on NHE3 activity in BBMV, we determined whether β/γ subunits were involved in the α₂-adrenergic agonist antagonism of D₁-like agonist inhibition of NHE3 activity. Antibodies against βcomm, slightly decreased NHE3 activity in the basal state (compared with the vehicle control or heat-denatured anti-βcomm antibody control) but had an insignificant effect on the inhibition of NHE3 activity by the D₁-like agonist (compared to D₁-like agonist alone, control group; Fig. 4A). All groups were compared with a vehicle control and to another control consisting of heat-denatured anti-βcomm (1:100 dilution) antibodies in place of nondenatured antibodies to
ensure the same ion and protein concentrations between groups (Fig. 4, A-C). However, anti-β_common antibodies (1:100 dilution) attenuated the α2-adrenergic agonist UK-14304 (5 × 10^{-10} M) reversal of the inhibitory action the D1-like agonist SKF-81297 (5 × 10^{-6} M, n = 4 (71 ± 5%), 5 × 10^{-8} M, n = 2 (75% ± 5%)) (Fig. 4B). The anti-β_common antibody used in these studies has been shown to be specific (13, 34). In the current report, the anti-β_common antibody recognized a 35-kDa protein in immunoblots of BBM (see below). β/γ Subunits were also involved in the diminished inhibitory effect of the D1-like agonist on NHE3 activity caused by PTX, because anti-β_common antibodies enhanced the inhibitory effect of SKF-81297 after PTX treatment of BBM (Fig. 4C).

To determine the mechanism of the β/γ-dependent effect of PTX treatment of BBM on D1 receptor-mediated NHE3 inhibition, the G_{a6}, G_{a6}, and β subunits were immunoprecipitated with their respective antibodies from vehicle- and PTX-treated BBM. The immunoprecipitates were electrophoresed and then immunoblotted using the antibody for β subunits (Fig. 5). There was no difference in the amount of β subunits (n = 5) immunoprecipitated in the control and PTX-treated BBM. PTX treatment tended to increase the amount of β/γ dimers associated with G_{a6} subunits (n = 5) and significantly decreased the amount of β/γ dimers associated with G_{a6} (n = 3).

There is physical linkage between NHE3 and G proteins. Three sets of studies were performed to determine the linkage between NHE3 and G_{a6}. In the first set of studies, renal BBMs were treated with vehicle, GTPγS, or GDPβS. The BBMs were then immunoprecipitated with anti-NHE3 antibody and immunoblotted with anti-G_{a6} antibody. Figure 6A demonstrates two bands (45 and 52 kDa) that represent G_{a6} observed after immunoblotting rat renal BBM with the anti-G_{a6} antibody. The amount of G_{a6} that immunoprecipitated with NHE3 was increased by GTPγS treatment [Fig. 6A, compare lanes 2 and 7, the GTPγS-treated BBM, with lanes 2 and 3, the vehicle (control)-treated

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**Fig. 6.** Regulated linkage between NHE3 and G_{a6}. A: Immunoblots of NHE3 and G_{a6}. In the first set of studies, immunoprecipitation was performed using anti-NHE3 antibodies and immunoblotted with anti-G_{a6} antibodies. BBMs from rat renal cortex were treated with vehicle (lanes 2 and 3), guanosine 5′-O-(2-thiodiphosphate) (GDPβS; 10^{-3} M, lanes 4 and 5), or guanosine 5′-O-(3-thiotriphosphate) (GTPγS; 10^{-3} M, lanes 6 and 7) for 30 min and immunoprecipitated with anti-NHE3 antibodies and immunoblotted with anti-G_{a6} antibodies. Lane 1 is immunoblot for G_{a6} showing 2 molecular sizes, 45 and 52 kDa. The densitometric analysis from 3 other studies is shown in B. In a second set of studies, immunoprecipitation was performed using anti-G_{a6} antibodies and immunoblotted with anti-NHE3 antibodies. Membranes or cells from immortalized renal proximal tubule cells (lanes 11–13) were treated with vehicle alone (lane 11), GTPγS (10^{-3} M; lane 12), or GDPβS (10^{-3} M; lane 13). The densitometric analysis from 3 other studies is shown in B. Linkage between NHE3 and G_{a6} is specific, because no band of the appropriate size (85 kDa) was seen when immunoprecipitant was IgG (lane 8). Moreover, a band of the appropriate size (85 kDa) was seen when membranes were immunoprecipitated with anti-NHE3 antibody and then immunoblotted with anti-NHE3 antibody (lane 9); this band corresponded to NHE3 band seen after immunoblotting of renal tubular cell membranes with anti-NHE3 antibody (lane 10). In a third set of studies, immortalized renal proximal tubule cells were incubated for 10 min with vehicle alone (lane 15) or the D1 receptor agonist fenoldopam (5 × 10^{-6} M; lanes 14 and 16). The whole cell lysates were immunoprecipitated with anti-G_{a6} antibodies (lanes 14 and 16) and immunoblotted with anti-NHE3 antibodies (lanes 14–16). Densitometric analyses are shown in B. Specificity of immunoprecipitate is shown by absence of immunoblotting of NHE3 using normal rabbit IgG as the immunoprecipitant (lane 14). B: Densitometric analyses of effect of GTPγS (10^{-3} M) and GDPβS (10^{-3} M) on amount of NHE3 linked to G_{a6} in renal BBM (n = 5/group). Effect of GTPγS (10^{-3} M) (n = 5), GDPβS (10^{-3} M) (n = 5), and fenoldopam (5 × 10^{-6} M) (n = 3) on amount of NHE3 linked to G_{a6} treatment of renal proximal tubule cells before membrane lysis. *P < 0.05 vs. respective controls, ANOVA for repeated measures, Scheffé’s test or paired t-test when only 2 groups were compared (e.g., control vs. fenoldopam-treated cells).
BBM). In contrast, the inactive analog of GDP, GDPβS (Fig. 6A, lanes 4 and 5), had no effect [Fig. 6A, compare lanes 2 and 3, the vehicle-treated BBM, (control), lanes 4 and 5, the GDPβS-treated BBM (negative control), and lanes 6 and 7, the GTPγS-treated BBM]. We have reported that GTPγS but not GDPβS enhanced the inhibitory effect of D1-like agonists on NHE3 activity in BBMV (12). The densitometric analyses of three other Gα studies are shown in Fig. 6B.

In the second set of studies, cell membranes from immortalized renal proximal tubule cells were immunoprecipitated with anti-Gα antibody and then immunoblotted with anti-NHE3 antibody. The reversal of the immunoprecipitation/immunoblotting antibody (compared with the first set of experiments) was performed to demonstrate the specificity of Gα/NHE3 coupling. Incubation of cell membranes from immortalized proximal tubule cells of WKY rats with GTPγS increased the amount of NHE3 linked to Gα (Fig. 6A, lane 12, the GTPγS-treated membranes, vs. lane 11, the vehicle-treated membranes). The inactive analog of GDP, GDPβS, did not affect the amount of NHE3 linked with Gα (Fig. 6A, lane 13, the GDPβS-treated membranes, vs. lane 12, the GTPγS-treated membranes, or lane 11, the vehicle-treated membranes). The densitometric analyses of three other studies are shown in Fig. 6B.

The linkage between NHE3 and Gα was specific because no band of the appropriate size (85 kDa) was seen in the control using rabbit IgG as the immunoprecipitant (Fig. 6A, lane 8). However, in an additional control for specificity, a band of the appropriate size (85 kDa) was seen when the membranes were immunoprecipitated with anti-NHE3 antibody and then immunoblotted with anti-NHE3 antibody (Fig. 6A, lane 9); this band corresponded to the NHE3 band seen after immunoblotting renal proximal tubular cell membranes with anti-NHE3 antibody (Fig. 6A, lane 10). Differences in sizes between lanes 9, 12, and 16 (immunoprecipitation) from lane 10 (immunoblot) were small and may be due to the persistent partial association of immunocomplex fragments with the immunoprecipitated NHE3 protein.

In the third set of studies, immortalized renal proximal tubule cells were incubated with the D1-like agonist fenoldopam for 10 min before cell lysis. These studies were performed to determine if Gα binds to NHE3 in the intact cell where cytoplasmic second messengers can be generated. The cell lysates were immunoprecipitated with anti-Gα antibody and then immunoblotted with anti-NHE3 antibody. Treatment of immortalized proximal tubule cells from WKY rats with the D1-like agonist fenoldopam (5 × 10⁻⁶ M for 10 min) increased the amount of NHE3 linked to Gα (Fig. 6A, lane 16, the fenoldopam-treated cells, vs. lane 15, the vehicle control-treated cells); the results were specific because no band of the appropriate size was seen when the lysates were immunoprecipitated with a rabbit IgG control instead of anti-Gα antibody (Fig. 6A, lane 14). The densitometric analyses of these studies are shown in Fig. 6B.

**DISCUSSION**

NHE3 activity is regulated by phosphorylation/dephosphorylation processes and membrane recycling in intact cells (14, 23, 42, 43). NHE3 activity can also be regulated by G proteins independent of cytoplasmic second messengers, but the G protein subunits involved in this regulation have not been reported (5, 10, 17). We now demonstrate a physical linkage between NHE3 and Gα and that this coupling increases after activation of regulatory pathways known to inhibit NHE3 activity in both rat renal BBMV and in the intact renal proximal tubular cell. In addition, we show that NHE3 activity can be regulated also by Gβγ dimers.

Activation of G protein-coupled receptors, e.g., D1-like receptors, by its agonist results in the mobilization of G protein subunits that may then interact with effectors, including enzymes (adenylyl cyclase, phospholipases) (37), ion channels (25), or transporter proteins (5, 10, 32). In keeping with the linkage of D1-like receptors and adenylyl cyclase activation, the immunoprecipitation studies demonstrated that the Gα subunit couples with NHE3 in a regulatory manner in intact cells (12). PKA has been shown to inhibit NHE3 activity whether naturally or heterologously expressed in kidney and other cell lines (12, 18, 39). PKC has also been reported to decrease NHE3 activity (18). However, the inhibitory action of dopamine on renal proximal tubular luminal NHE3 activity is mediated by PKA but not by phospholipase C signal transduction products (11, 12, 32). Eicosanoids generated from cytochrome P-450 metabolism may also participate in the regulation of NHE activity in BBMV (27, 32). In the current study, the inhibitory effect of the D1-like receptor on NHE3 activity in rat renal BBMV was not affected by ETYA (data not shown), an inhibitor of arachidonic acid metabolism. Dopamine can also inhibit NHE1 activity (26). However, expression and activity of NHE1, which is found in basolateral membranes, was not detected in our BBM preparation. There are no other NHE isoforms in BBMs of renal proximal tubules; indeed the NHE isoform responsible for sodium transport in the BBM of renal proximal tubules has been reported to be NHE3 (1, 2, 4, 41).

Dopamine, via D1-like receptors, has also been shown to inhibit NHE activity in rat renal proximal tubules independently of PKA (10, 32) and PKC (10); this second messenger-independent regulation of NHE activity is G protein linked (10, 32). The current studies confirm and extend these observations. With the use of renal BBMVs that are devoid of cytoplasmic second messengers and adenosine triphosphate (10), we find that dopamine and two different D1-like agonists inhibit NHE3 activity in a concentration-dependent manner. In the current report, we determined which G protein subunit is linked to the inhibitory effect of D1-like receptors on NHE3 activity in BBMV independent of cytoplasmic second messengers. Immunoprecipitation studies using proximal tubule cell membranes and rat renal BBM demonstrated that stimulation of D1-like receptors or direct activation of the heterotri-
meric G proteins with GTPγS resulted in a robust increase in the amount of Gαs associated with the NHE3. In addition, anti-Gαα antibodies reduced the D1-like receptor agonist-mediated NHE3 inhibition. The mechanism by which PTX treatment decreased the inhibitory effect of the D1-like agonist on NHE3 activity is probably an indirect one. The stabilization of Gαβγ heterotrimer by PTX (16) may have limited the amount of βγ dimers available to form heterotrimer with Gαα and thereby decreased its coupling to D1-like receptors. At any rate, these studies provide concrete evidence of a direct regulatory role for Gαα, independent of second messengers, in the D1-like-mediated inhibition of NHE3 activity in rat renal BBMV.

The Gαα subunit had no discernible role in the regulation of basal or D1-like agonist-mediated inhibition of NHE3 activity in BBMV. The α2-adrenergic agonist, by itself, did not affect NHE3 activity, an action that was not modified by PTX. However, we confirmed that α2-adrenergic agonist stimulation counteracted the inhibitory effect of a D1-like agonist on NHE3 activity in BBMV. The modest α2-adrenergic receptor agonist-mediated reversal (22–55%) of the D1-like receptor-mediated inhibition may be related to the lower expression of α2-adrenergic receptors relative to D1-like receptors in rat renal BBM [D1-like receptor maximal binding (Bmax) = 206 fmol/mg protein, α2-adrenergic receptor Bmax = 108 fmol/mg protein] (15, 36). UK-14304 exerts its actions via α2-adrenergic receptors, and, under physiological conditions, the α2-adrenergic receptor is linked to Gαα (8). UK-14304 has been shown to stimulate NHE3 activity via α2-adrenergic receptors/ Gαα in intact opossum kidney cells (8). Because α2-adrenergic receptors are linked to Gαα, the reversal of the inhibitory action of the D1-like agonist should have been blocked by antibodies to Gαα; G1αt is the most abundant Gα subunit isomorph in renal proximal tubules (35). However, the antibodies to G1αt did not affect the ability of an α2-adrenergic agonist to counteract the inhibitory effect of a D1-agonist on NHE3 activity. The inability of anti-G1αt antibodies to affect NHE3 activity was not due to the presence of other isoforms of the Gαα subunit, because PTX treatment of BBM did not affect basal NHE3 activity or alter any α2-adrenergic agonist effect. Thus the PTX and the anti-G1αt antibody studies suggest that Gαα did not mediate the ability of the α2-adrenergic agonist to attenuate the inhibitory action of D1-like receptors on NHE3 activity in BBMV. Taken together, the failure of PTX and anti-Gαα antibodies to reduce the α2-adrenergic agonist effect suggests a model that includes a PTX-insensitive G protein. In light of the results obtained using the anti-Gαaa antibody (discussed below), this pathway appears to involve release of βγ subunits, which preferentially couple an as yet unidentified heterotrimeric G protein.

Because activation of Gαα could not explain the ability of the α2-adrenergic receptor agonist to reverse the inhibitory effect of the D1-like agonist on NHE3 activity in BBMV, we turned our attention to the G protein βγ subunits. In BBM, antibodies to βcommon alone had a small but significant effect on basal NHE3 activity, which suggests that βγ dimers have a tonic stimulatory effect on NHE3 activity. The lack of a substantial effect of anti-βcommon antibodies in the D1-like receptor inhibition of NHE3 activity is likely due to the fact that maximum NHE3 inhibition by the D1-like agonist has already been achieved. In addition, the inhibitory effect of Gαα may normally predominate over the stimulatory effect of βγ dimers. However, antibodies to βcommon reversed the ability of the α2-adrenergic agonist to attenuate the inhibitory effect of the D1-like agonist. These data implicate βγ dimers in the effect of PTX on D1-like receptor action. However, the effect of PTX seen here is difficult to rationalize according to present models of PTX action, in which it is seen as an uncoupler of receptors and their respective subclass of G proteins (16). Nevertheless, the functional effect of PTX treatment of BBMs and the reversal of this effect by anti-β antibodies were highly reproducible in this experimental system. The most economical interpretation of these results would be that βγ dimers released from a pool of PTX-insensitive G proteins by the α2-adrenergic agonist bind and stimulate NHE3 activity, opposing the effect of Gαα. Moreover, this model is consistent with preliminary studies from our laboratory suggesting that NHE3 can physically couple to Gβγ (24).

The mechanism by which PTX treatment is able to counteract the inhibitory effect of D1-like agonists on NHE3 function is more obscure. Certainly this must be an indirect effect, because PTX is unable to modify Gαα directly. A clue to a possible mechanism emerged from the immunoprecipitation data of Fig. 5, where PTX treatment of BBMs and the reversal of this effect by anti-β antibodies were highly reproducible in this experimental system. The most economical interpretation of these results would be that βγ dimers released from a pool of PTX-insensitive G proteins by the α2-adrenergic agonist bind and stimulate NHE3 activity, opposing the effect of Gαα. Moreover, this model is consistent with preliminary studies from our laboratory suggesting that NHE3 can physically couple to Gβγ (24).

In summary, D1-like agonists via Gαα, independent of cytoplasmic second messengers, inhibit NHE3 activity in what appears to be a direct regulatory coupling of the two proteins. βγ dimers released on activation of α2-adrenergic receptors can act to oppose the Gαα inhibitory effect most likely at the level of NHE3. Our findings do not rule out that PTX-insensitive Gα subunits implicated here in α2-adrenergic signaling may also contribute to stimulation of NHE3. Further re-
search will also be required to determine if separate functional pools of Gβγ, observed in intact cells (20), exert regulatory effects both at the level of GαA and the effector NHE3. Proteins that regulate NHE3 activity, such as NHE regulatory factor and NHE3 kinase A regulatory protein, are unlikely to be involved in our experiments using BBMV, because the regulation of NHE3 by these proteins involves cAMP (14).

**Perspectives**

Dopamine, via D2-like receptors, inhibits NHE3 activity via PKA-dependent and PKA-independent pathways. GαA can directly inhibit whereas Gβγ can indirectly and/or directly stimulate NHE3 activity. PTX-insensitive Gαa subunits (Gα12 and Gα13, or Gαi1), instead of Gβγ, could also mediate the stimulation of NHE3 activity in BBMV. Gα12 has been shown to stimulate NHE3 activity in cell lines heterologously expressing this isoform (26). It is also possible that the G protein-dependent, PKA-independent pathway is an initial step that precedes second messengers in the transduction of the receptor signal.

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