THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 286, NO. 22, pp. 19259–19269, June 3, 2011

Regulation of the Epithelial Na^+ Channel by the RH Domain of G Protein-coupled Receptor Kinase, GRK2, and $G\alpha q/11$*

Received for publication, March 14, 2011, and in revised form, March 31, 2011 Published, JBC Papers in Press, April 4, 2011, DOI 10.1074/jbc.M111.239772

Il-Ha Lee[‡], Sung-Hee Song[‡], Craig R. Campbell[‡], Sharad Kumar[§1], David I. Cook[‡], and Anuwat Dinudom[‡2]

From the [‡]Discipline of Physiology, The Bosch Institute, Faculty of Medicine, The University of Sydney, NSW 2006, Australia and the [§]Centre for Cancer Biology, SA Pathology, Adelaide, SA 5000, Australia

The G protein-coupled receptor kinase (GRK2) belongs to a family of protein kinases that phosphorylates agonist-activated G protein-coupled receptors, leading to G protein-receptor uncoupling and termination of G protein signaling. GRK2 also contains a regulator of G protein signaling homology (RH) domain, which selectively interacts with α-subunits of the Gq/11 family that are released during G protein-coupled receptor activation. We have previously reported that kinase activity of GRK2 up-regulates activity of the epithelial sodium channel (ENaC) in a Na^+ absorptive epithelium by blocking Nedd4-2-dependent inhibition of ENaC. In the present study, we report that GRK2 also regulates ENaC by a mechanism that does not depend on its kinase activity. We show that a wild-type GRK2 (wtGRK2) and a kinase-dead GRK2 mutant (^{K220R}GRK2), but not a GRK2 mutant that lacks the C-terminal RH domain (ΔRH-GRK2) or a GRK2 mutant that cannot interact with $G\alpha q/11/14$ (^{D110A}GRK2), increase activity of ENaC. GRK2 up-regulates the basal activity of the channel as a consequence of its RH domain binding the α-subunits of Gq/11. We further found that expression of constitutively active $G\alpha q/11$ mutants significantly inhibits activity of ENaC. Conversely, co-expression of siRNA against $G\alpha q/11$ increases ENaC activity. The effect of $G\alpha q$ on ENaC activity is not due to change in ENaC membrane expression and is independent of Nedd4-2. These findings reveal a novel mechanism by which GRK2 and Gq/11 α-subunits regulate the activity ENaC.

The β-adrenergic receptor kinase 1 (GRK2) is one of the G protein-coupled receptor serine/threonine kinases (GRKs).[3] All seven members of the GRK family (GRK1–7) share a highly homologous kinase domain that is flanked toward the C-terminal by a pleckstrin homology (PH) domain and toward the N-terminal by a regulator of G-protein signaling homology (RH) domain (1). Protein kinases of this family are unique in their ability to specifically phosphorylate the agonist-activated form of heptahelical G protein-coupled receptors (GPCRs) (2). Upon stimulation, GRKs facilitate binding of agonist-activated GPCRs to cytosolic cofactor proteins, arrestins, and other proteins involved in receptor desensitization (3, 4). This interaction impairs coupling between the GPCRs and trimeric G proteins, targets GPCRs for clathrin-mediated endocytosis (3), and terminates G protein signal transduction (2). Activity of GRKs is, therefore, important for arbitrating an appropriate strength and duration of cellular responses, allowing the G protein-dependent cellular responses to physiological stimulation to cease rapidly after receptor activation even in the continuing presence of stimuli.

Recent studies suggest that, in addition to their ability to inactivate GPCRs by phosphorylation, GRKs can phosphorylate and regulate activity of an array of non-receptor substrates (2) and also regulate GPCR signaling by a mechanism that does not require their intrinsic kinase activity (3). For instance, the RH domain of GRK2 contains a binding site that selectively interacts with the α-subunits ($G\alpha$) of the trimeric G proteins Gq, G11, and G14, but excludes $G\alpha$ of other Gq/11 family members and those of Gi, Go, or Gs (3, 5–7). Binding of GRK2 to $G\alpha q/11$ effectively terminates transduction of the $G\alpha$ signal (3, 5–7). This phosphorylation-independent mechanism has now been implicated in the regulation of several Gq/11-coupled receptor signaling events (2, 8, 9).

Amiloride-sensitive epithelial Na^+ channels (ENaC) play an important role in regulating Na^+ and fluid homeostasis, maintaining blood pressure (10) and regulating volume of the alveolar fluid (11). ENaC is expressed in a variety of tissues including the renal collecting duct, the distal colon, the lungs and the ducts of exocrine glands. Activity of ENaC in these tissues is regulated by an array of physiological factors including hormones, nucleotides, and cytosolic ion concentrations, many of which are known to exert their effect on the channel by mechanisms that involve trimeric G proteins (12–16). We have previously reported, in salivary duct cells, that ENaC is maintained in an active state by the kinase activity of GRK2 and that GRK2 inhibits Na^+ feedback inhibition of ENaC (17). Na^+ feedback inhibition of ENaC involves the α-subunit of the G protein, Go (13), and Nedd4 (14) and/or Nedd4-2 (18), the ubiquitin protein ligases, which play an important role in internalization of ENaC (19–21) and regulate membrane expression of the channel. Given that GRK2 phosphorylates the C-terminal of the β-subunit of ENaC (17) and Nedd4 and Nedd4-2 (22), this phosphorylation-dependent effect of GRK2 on ENaC might

* This work was supported by grants from the National Health and Medical Research Council of Australia (508086) and the Australian Research Council (DP0774320).

[1] An NH&MRC Senior Principal Research Fellow.

[2] An NH&MRC Senior Research Fellow. To whom correspondence should be addressed: Medical Foundation Building, 92-94 Parramatta Rd., Camperdown, NSW 2050, Sydney, Australia. Tel.: 61-2-9036-3317; Fax: 61-2-9036-3316; E-mail: anuwat@physiol.usyd.edu.au.

[3] The abbreviations used are: GRK, G protein-coupled receptor kinase; RH, regulator of G protein signaling homology; ENaC, epithelial Na^+ channel; Nedd4, neural precursor cell expressed developmentally down-regulated protein 4; PY motif, proline-rich motif; $G\alpha$, G protein α-subunit; Isc, equivalent short-circuit current; PLCβ, phospholipase Cβ; BIM, bisindolylmaleimide-1; PIP_2, phosphatidylinositol 4,5-bisphosphate.

involve multiple phosphorylated effector targets, which are not GPCRs.

The present study was inspired by our observation that a GRK2 mutant that lacks kinase activity was effective in up-regulating the basal activity of ENaC. We found that this stimulatory effect of GRK2 on ENaC can be attributed to the ability of the RH domain of GRK2 to interact with and inhibit the α-subunit of Gq/11, which acts as a negative regulator of ENaC.

EXPERIMENTAL PROCEDURES

DNA Constructs—Wild-type mouse α-, β- and γ-ENaC and α-ENaC clones containing HA at the N-terminal and V5 at the C-terminal, all of which are in pcDNA3.1, were a gift from Thomas R. Kleyman (University of Pittsburgh). α-, β-, and γ-ENaC subclones containing FLAG at the C-terminal in pcDNA3.1 were provided by Angeles Sanchez-Perez (University of Sydney, Australia). WtGRK2, ^{K220R}GRK2, ^{D110A}GRK2, RH domain of GRK2, wtGαq, and ^{Q209L}Gαq in pcDNA3 were gifts from Philip B. Wedegaertner (Thomas Jefferson University). ΔRH-GRK2 in pcDNA3.1 was a gift from Jerrold M. Olefsky (University of California). ^{Q209L}Gα11, ^{Q205L}Gα14, and ^{Q212L}Gα15/16 in pcDNA3.1 were purchased from cDNA Resource Center (University of Missouri). The C-terminal-truncated ENaC mutants were generated by PCR-based methods performed using primers, which have stop codons at specific sites to generate Pro^{646}stop α-ENaC, Cys^{594}stop β-ENaC or Phe^{610}stop γ-ENaC.

Cell Culture and Transfection—Fischer rat thyroid (FRT) cells were a gift from Lucio Nitsch (University of Naples), M1 mouse collecting duct cells, originally generated by Stoos *et al.* (23), were a gift from Christoph Korbmacher (Universität Erlangen-Nürnberg, Germany) and HEK293T cells were purchased from the American Type Culture Collection. These cell types were cultured in Dulbecco's modified Eagle's/Ham's F-12 media with 100 units/ml penicillin and 100 μg/ml streptomycin at 37 °C. The media for FRT cells contained 5% fetal bovine serum, the media for HEK293T cells contained 10% fetal bovine serum whereas the media for M1 cells contained 10% fetal bovine serum and 100 nM dexamethasone. FRT and M1 cells were seeded onto Millicell-PCF permeable filter supports (Millipore, catalogue number PIHP01250). One day after seeding, FRT cells were co-transfected with cDNA of α-, β-, and γ-mENaC-FLAG (0.7 μg/ml each). When appropriate, FRT and M1 cells were transfected with cDNA of wtGRK2, mutants of GRK2, mutants of Gαq, Gα11 or Gα14 (3 μg/ml), siRNA against GRK2, siRNA against Nedd4-2 or siRNA against Gαq, Gα11, or Gα14 (0.5 μg/ml). siRNAs against GRK2 (5′-GGA CAC AAA AGG AAT CAA GTT-3′) and Gαq (5′-GGC TCA TGC ACA ATT GGT-3′) were obtained from Ambion and siRNAs against Gα11 (5′-CAC AAC TGG CAT CAT CGA GTA-3′), Gα14 (5′-TTG GTT GCT CTG AGT GAA TAT-3′) and Nedd4-2 (5′-AAC CAC AAC ACA AAG TCA CAG-3′) were obtained from Qiagen. In short, cDNA or siRNA were mixed with Lipofectamine-2000 (Invitrogen) in Opti-MEM reduced serum media (Invitrogen) and incubated for 20 min at room temperature before being transferred to the apical side of the monolayer and further incubated for 4 h at 37 °C. The transfection medium was then replaced with Dulbecco's modified Eagle's/Ham's F-12 media containing fetal bovine serum and antibiotics. In addition, the medium also contained dexamethasone (100 nM) for M1 cells or amiloride (10 μM) for FRT cells.

RT-PCR and Quantitative RT-PCR—Total RNA was isolated from FRT cells using TRI Reagent® (Sigma). 2 μg of total RNA was used for reverse transcription (RT) reactions. The following primers were used: Gα11 (forward, 5′-GAC CAG AAC AAG GCC AAT GC-3′; reverse, 5′-GAA GAT GAT GTT CTC CAG GTC-3′), Gα14 (forward, 5′-CAG AAT AAG GAA AAT GCC CAG-3′; reverse, 5′-CCT GGT CAT ATT CAC TCA GAG-3′), and GAPDH (forward, 5′-TGG CCT TCC GTG TTC CTA CC-3′; reverse, 5′-TGT AGG CCA TGA GGT CCA CCA C-3′). PCR amplification was performed for 30 cycles. A 10 μl aliquot of the PCR product was then separated by electrophoresis on a 1.5% agarose gel and stained with ethidium bromide. Quantitative RT-PCR (QRT-PCR) reactions of newly synthesized RT products were carried out using KAPA SYBR FAST qPCR Kit (Kapabiosystems). Fluorescence detection was performed using the RG-3000 real-time PCR system (Corbett Research). Amplification conditions consisted of 40 cycles of 95 °C for 15 s and 60 °C for 1 min after incubation at 95 °C for 5 min for enzyme activation. Reaction efficiencies and relative gene expression were analyzed using REST 2009 software program from Qiagen.

Immunoblot—Two days after transfection, cells were washed twice with phosphate-buffered saline before treatment with a lysis buffer containing (in mM) Tris-HCl (50), NaCl (150), EDTA (10), with 10% glycerol and 1% Triton X-100 plus Complete Protease Inhibitor mixture (Roche Applied Science). After the protein concentration of each lysate had been determined, equal amounts of protein lysate were loaded onto a 12% SDS-polyacrylamide gel. Following electrophoresis, the protein was transferred to a nitrocellulose membrane and incubated overnight with anti-GRK2 monoclonal antibody, anti-Gαq polyclonal antibody, or anti-β-actin monoclonal antibody (Santa Cruz Biotechnology), anti-FLAG M2 monoclonal antibody (Sigma), or anti-HA monoclonal antibody (Cell Signaling). The blots were washed to remove unbound antibodies before incubation with a horseradish peroxidase-conjugated secondary antibody for 1 h. The blots were washed with a TBS buffer containing 0.1% Tween-20. The proteins of interest were then visualized using an ECL™ Western blotting kit (GE Healthcare) and quantitated by densitometric analysis using ImageJ (NIH). The data are representative of at least three experiments.

Quantitation of Amiloride-sensitive Na^+ Current—After the monolayers became confluent, normally within 2–3 days after transfection, the Millicell-PCF insert was transferred to a modified Ussing chamber. Apical and basolateral surfaces of the monolayer were simultaneously perfused with a solution containing (in mM) NaCl (130), $CaCl_2$ (1), KCl (1), $MgCl_2$ (1), glucose (5), HEPES (10), pH 7.4, maintained at 37 °C. The chamber was connected to a VCC MC6 multichannel voltage/current clamp amplifier, controlled and monitored using the Acquire & Analyze software (V2.3.177, Physiologic Instruments, San Diego, CA). Experiments were carried out under current clamp (open circuit) conditions as previously described (24). Transepithelial potential was measured with reference to the baso-

lateral side of the epithelium and current pulses were injected to assess the transepithelial resistance. The equivalent short-circuit current was calculated using Ohm's law and plotted by the software. Amiloride-sensitive equivalent short-circuit current (I_{ami}) was determined as the change in current following the addition of amiloride (10 μM) to the apical bathing solution. Because of large variation of I_{ami} between each batch of cells, all data were normalized by dividing the amiloride-sensitive short circuit current of each experiment by an average of the amiloride-sensitive short circuit current of at least 3 control experiments obtained from the same batch of cells in the same day. This ratio is reported as normalized amiloride-sensitive equivalent short-circuit current ($I_{ami(normalized)}$). The mean value of I_{ami} of each control group is reported in the figure legends. Data for each experiment were obtained from at least three different batches of cells and are reported as mean ± S.E. with the number of experiments in parentheses. Statistical significance was assessed using Student's *t* test.

Quantitation of Na^+/K^+ ATPase Activity—M1 cell monolayers were mounted in a modified Ussing chamber bathed symmetrically with the physiological solution and the equivalent short-circuit current monitored. Activity of the Na^+/K^+-ATPase was determined as previously described (24). In short, amiloride (10 μM) was added to the apical bathing solution to inhibit activity of ENaC. Nystatin (360 μg/ml) was then added to the apical solution to permeabilize the apical membrane and to eradicate any rate limitation associated with the apical Na^+ entry. Short circuit current (I_{sc}) was allowed to stabilize for 30 min before the addition of 1 mM ouabain, an inhibitor of the Na^+/K^+-ATPase, to the basolateral solution. The change in I_{sc} following the addition of ouabain was used to estimate the activity of the Na^+/K^+-ATPase.

Surface Expression of ENaC—HEK293 cells were transfected with FLAG-tagged α-, β-, and γ-mENaC. Two days after transfection, the cells were washed three times with ice-cold phosphate-buffered saline and then incubated for 30 min in 5 ml of cell-impermeant sulfo-NHS-SS-biotin solution (0.5 mg/ml; Pierce) at 4 °C. The reaction was stopped by quenching with Tris-buffered saline. The cells were solubilized in lysis buffer, and the lysate centrifuged at 14,000 rpm for 5 min at 4 °C. The supernatant was collected and mixed with 250 μl of NeutrAvidin™ gel slurry (Pierce) before incubating, with gentle rocking, for 60 min at room temperature. After incubation, the sample was centrifuged at 1,000 rpm for 2 min. The precipitant containing the biotinylated proteins was washed five times with lysis buffer. Finally, the biotinylated proteins were eluted by the addition of SDS sample buffer and then analyzed by Western blot using anti-FLAG M2 monoclonal antibody or anti-HA monoclonal antibody.

RESULTS

GRK2 Activates ENaC by a Phosphorylation-independent Mechanism—Our previous study in isolated mouse mandibular duct cells suggested that kinase activity of GRK2 up-regulates activity of ENaC and prevents down-regulation of the channel by Na^+ feedback inhibition (17). To further investigate the role of GRK2 in regulation of ENaC activity, we transiently expressed GRK2 in mouse renal collecting duct (M1) cells that

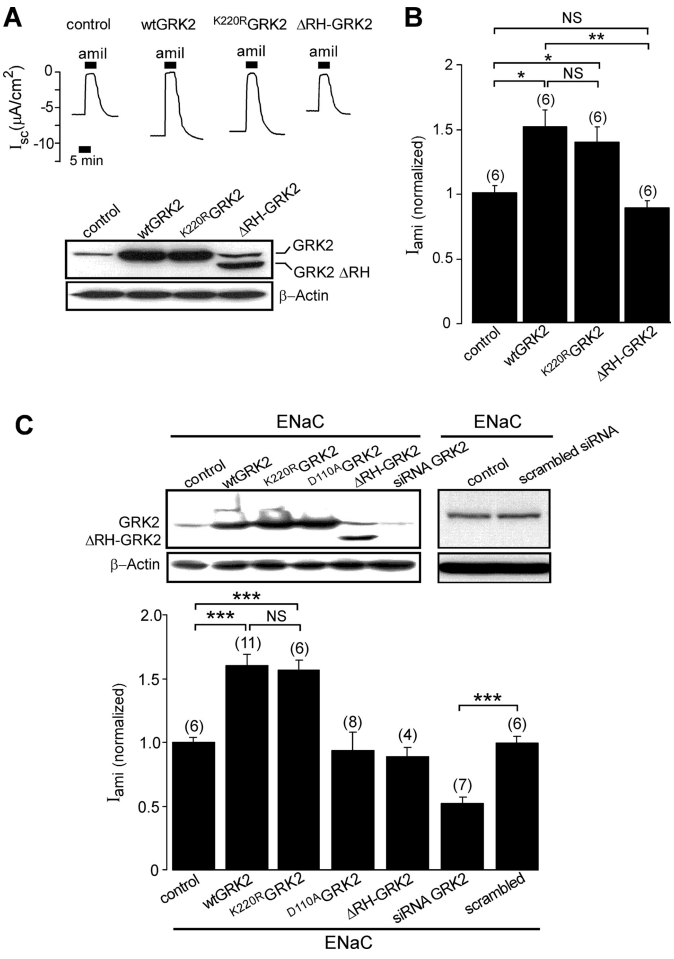

FIGURE 1. **GRK2 regulates ENaC activity.** *A (upper panel)*, representative recordings of equivalent short-circuit current (I_{sc}) in untransfected M1 cell monolayers (*control*) or monolayers expressing wtGRK2, a kinase-inactivated mutant (^{K220R}GRK2) or an RH domain deletion mutant of GRK2 (ΔRH-GRK2), showing the response to 10 μM amiloride (*solid bar*). *A* (*lower panel*), immunoblot analysis of expression of the wtGRK2, ^{K220R}GRK2 and ΔRH-GRK2 in M1 cells in experiments corresponding to the *upper panel*. β-Actin was used as a control protein. *B*, normalized amiloride-sensitive short-circuit current ($I_{ami\ (normalized)}$) in untransfected M1 cells (*control*) or cells transfected with wtGRK2, ^{K220R}GRK2 or ΔRH-GRK2 as in *A*. The average I_{ami} for the control group is 20.63 ± 2.37 μA/cm² ($n = 6$). *C*, immunoblot analysis (*upper panel*) and $I_{ami\ (normalized)}$ (*lower panel*) in FRT cells transfected with three subunits of ENaC (*control*) or co-transfected with ENaC and with wild-type GRK2 (wtGRK2), ^{K220R}GRK2, ^{D110A}GRK2, ΔRH-GRK2, siRNA against GRK2 or scrambled siRNA as negative control. The average I_{ami} for the control group is 17.42 ± 0.87 μA/cm² ($n = 6$). NS indicates no significant difference. *, **, and *** indicate $p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively. The number of experiments is shown in parentheses.

endogenously express ENaC by transfecting the cells with a plasmid containing wild-type GRK2 (wtGRK2) (Fig. 1*A*, *lower panel*). Activity of ENaC was determined from transfected cell monolayers in Ussing chamber experiments (Fig. 1, *A* and *B*). We found that over-expression of GRK2 significantly increased the normalized amiloride-sensitive Na^+ current (1.53 ± 0.17, $n = 6$ *versus* 1.00 ± 0.07, $n = 6$ in untransfected cells; $p < 0.05$), consistent with GRK2 positively regulating the activity of ENaC as reported in our previous study (17). We, therefore, assessed whether the effect of GRK2 on the basal activity of ENaC in M1 cells is mediated by its kinase activity. We did so by overexpressing a kinase-dead GRK2 mutant (^{K220R}GRK2) (Fig. 1*A*), in which the invariant lysine residue 220 in the protein kinase

catalytic domain was converted to an arginine to terminate kinase activity (25). We found that activity of ENaC in cells expressing the ^{K220R}GRK2 mutant (1.40 ± 0.14, $n = 6$) was significantly higher than that of the control group ($p < 0.05$) and that the effect of the ^{K220R}GRK2 mutant on ENaC was not significantly different from that of the wtGRK2 (Fig. 1*B*). Thus, the mechanism by which GRK2 stimulates the basal activity of ENaC is not associated with its kinase activity. Furthermore, expression of a GRK2 construct that lacks the N-terminal RH domain (ΔRH-GRK2) (26) was without any effect on the activity of ENaC (Fig. 1*B*). Together, these data suggest that the effect of GRK2 on the activity of ENaC in M1 collecting duct cells is independent of its action as a protein kinase but dependent on its RH domain.

To determine the mechanism by which the RH domain of GRK2 regulates ENaC, we first established a mammalian expression system that could be used for further investigation. We reconstituted ENaC in Fischer Rat Thyroid (FRT) cells by transiently expressing the α-, β-, and γ-ENaC subunits. A similar approach has previously been used successfully to investigate regulation of ENaC (24, 27). The cells were co-transfected with the wtGRK2 construct or with siRNA directed against GRK2 (Fig. 1*C*). In agreement with our studies in M1 cells (Fig. 1, *A* and *B*), overexpression of GRK2 enhanced normalized amiloride-sensitive current in FRT cells (1.63 ± 0.10, $n = 11$ *versus* 1.00 ± 0.05, $n = 6$ in control ENaC-only transfected cells; $p < 0.001$), while siRNA-mediated knockdown of GRK2 expression significantly attenuated the amiloride-sensitive current compared with that of cells transfected with a scrambled siRNA (0.53 ± 0.04, $n = 7$ *versus* 1.02 ± 0.05, $n = 6$; $p < 0.001$, Fig. 1*C*). In addition, the siRNA directed against GRK2 inhibited expression of endogenous GRK2 by 50% ($p < 0.05$), whereas the scrambled siRNA was without any affect. As observed in M1 cells, the effect of GRK2 on the activity of ENaC was independent of its kinase activity. We found that normalized amiloride-sensitive current in cells expressing the ^{K220R}GRK2 mutant (1.58 ± 0.09, $n = 6$) was significantly higher than that of the control group ($p < 0.001$). Expression of the ^{K220R}GRK2 mutant increased normalized amiloride-sensitive current in ENaC-transfected FRT cells to a value that is not significantly different from that of cells expressing wtGRK2 (Fig. 1*C*). Thus, kinase activity is not essential for GRK2 to exert its stimulatory effect on the activity of ENaC in FRT cells. To confirm the role of the RH domain, cells were co-transfected with ENaC and the ΔRH-GRK2 mutant (Fig. 1*C*). Under these conditions, the normalized amiloride-sensitive current (0.89 ± 0.09, $n = 4$) observed in the co-tranfected cells was not significantly different from that of the control cells expressing ENaC alone (1.00 ± 0.05, $n = 6$; $p > 0.05$). Subsequently, ENaC and a GRK2 mutant (^{D110A}GRK2), in which the aspartic acid at the position 110 in its RH domain was mutated to alanine, were co-expressed in FRT cells. This GRK2 mutant has been shown to be unable to interact with Gαq/11/14 (28, 29). We found that, the ^{D110A}GRK2 mutant failed to alter activity of ENaC (Fig. 1*C*), supporting the conclusion that the observed effect of GRK2 on ENaC may be due to GRK2 preventing Gαq from inhibiting the activity of ENaC.

Gq/11 Family Proteins Inhibit Activity of ENaC—We then investigated the effect of Gαq/11 on ENaC activity by suppressing expression of Gαq using an siRNA in FRT cells co-transfected with ENaC (Fig. 2*A*). We found that normalized amiloride-sensitive current in Gαq siRNA-transfected cells (1.25 ± 0.08, $n = 6$) was significantly higher than that of the control cells (1.00 ± 0.05, $n = 8$; $p < 0.01$). Next, FRT cells were co-transfected with ENaC and a constitutively active Gαq mutant, ^{Q209L}Gαq (30, 31), which decreased the normalized amiloride-sensitive current in FRT cells (0.15 ± 0.05, $n = 8$; $p < 0.001$; Fig. 2*A*), suggesting that Gαq is a negative regulator of the channel. We then confirmed these findings in M1 collecting duct cells, in which ENaC is endogenously expressed, by showing that over-expression of the constitutively active ^{Q209L}Gαq mutant abolished activity of ENaC and the siRNA directed against Gαq significantly increased activity of the channel (Fig. 2*B*).

To determine whether ENaC is also regulated by the α-subunits of Gα11 and Gα14, which are known to interact with GRK2, we expressed constitutively active ^{Q209L}Gα11 and ^{Q205L}Gα14 mutants in FRT cells. Expression of ^{Q209L}Gα11 and ^{Q205L}Gα14 was confirmed by RT-PCR (Fig. 2, *C* and *D*, *upper panel*). qPCR analysis data (Fig. 2, *C* and *D*, *lower panel*) suggest that relative expression of ^{Q209L}Gα11 and ^{Q205L}Gα14 was increased by 4–5 fold, whereas siRNAs decreased expression of their respective targets by over 80%. Moreover, we found that over-expression of ^{Q209L}Gα11 and ^{Q205L}Gα14 mutants inhibited the amiloride-sensitive Na^+ current (Fig. 2*E*). Conversely, siRNAs directed against Gα11 and Gα14 attenuated expression of Gα11 and Gα14 (Fig. 2, *C* and *E*) and significantly increased activity of ENaC (Fig. 2*E*). In addition, expression of the constitutively active ^{Q212L}Gα15/16 mutant also inhibited the amiloride-sensitive Na^+ current (data not shown). Hence, the Gα of all of the Gq/11 family act as negative-regulators of ENaC activity.

To eliminate a possibility that the inhibitory effect of Gq on ENaC activity may be due to Gq inhibiting Na^+/K^+-ATPase function, the activity of which is required for generating a driving force for Na^+ absorption via ENaC (32), we determined the effect of Gq on the ouabain-sensitive current which represents activity of the Na^+/K^+-ATPase. We found that expression of ^{Q209L}Gαq has no effect on the ouabain-sensitive current in M1 cells (Fig. 3, *A* and *B*).

It has been suggested that proteolysis of ENaC subunits by serine proteases is important for maturation and activation of ENaC (33–35). It is, therefore, conceivable that Gαq inhibits ENaC by attenuating proteolysis of the channel. To address this issue, we co-expressed, in HEK293T cells, an α-ENaC clone that has HA at the N-terminal and V5 at the C-terminal together with β- and γ-ENaC, which have FLAG at the C-terminal, and ^{Q209L}Gαq. It was previously shown that the α-ENaC is cleaved by serine proteases, yielding a 30 kDa N-terminal fragment (33). Our immunoblot analysis of the biotinylated membrane proteins, using anti-HA antibody, revealed the presence of N-terminal fragments of α-ENaC (Fig. 3*C* with control biotinylation in Fig. 3*E*). No biotinylated proteins were detected in the cytoplasmic fraction (Fig. 3*F*). A ratio between the density of the 30 kDa α-ENaC N-terminal fragment and that of 95 kDa

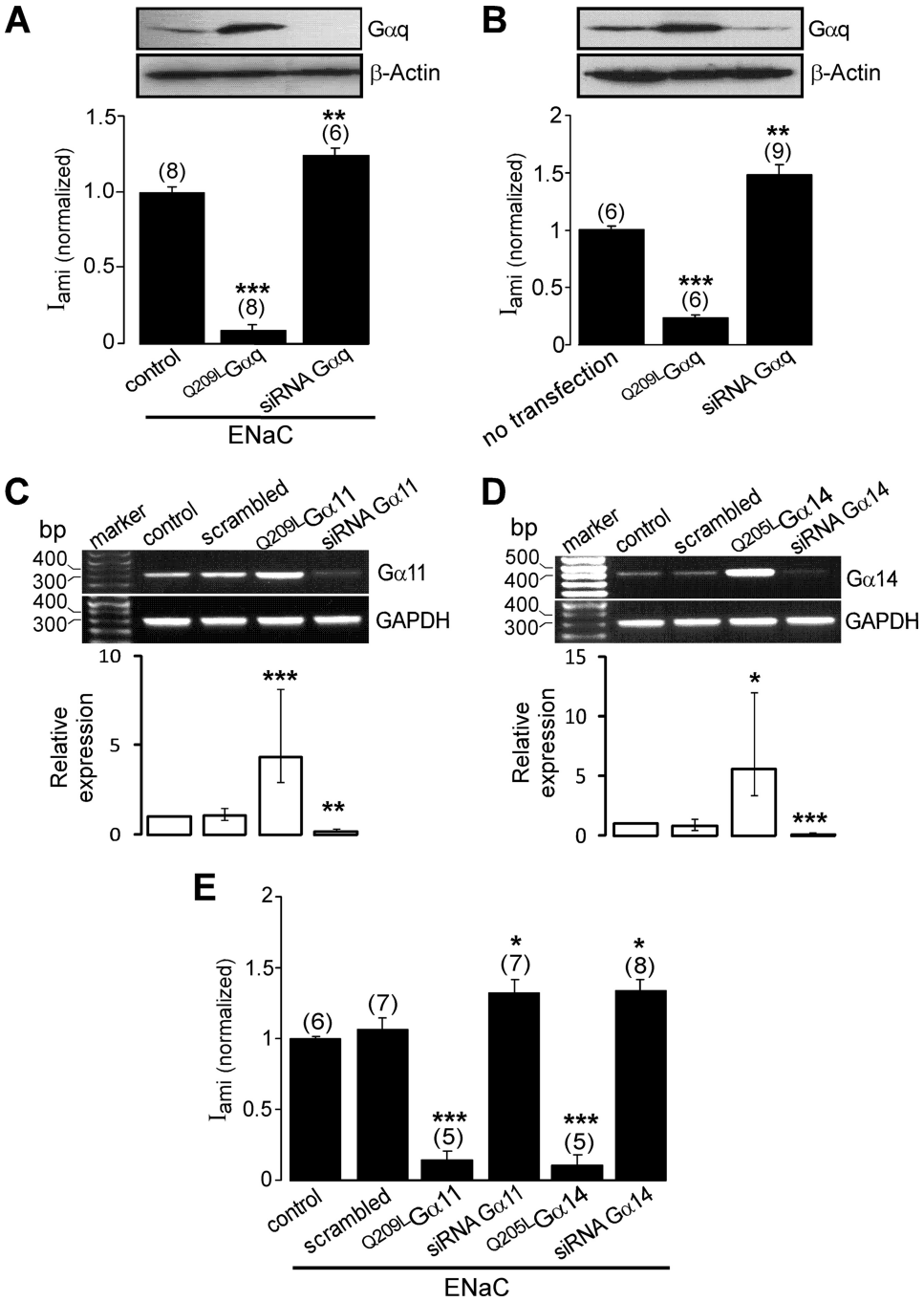

FIGURE 2. **Gαq decreases ENaC activity.** *A*, immunoblot analysis (*upper panel*) and $I_{ami\ (normalized)}$ (*lower panel*) of untransfected FRT cells (control) or FRT cells transfected with ^{Q209L}Gαq or siRNA directed against Gαq. β-Actin was used as control protein. The average I_{ami} for the control group is 4.69 ± 1.11 μA/cm^2 (n = 8). *B*, immunoblot analysis and the corresponding $I_{ami(normalized)}$ measured in M1 cells (*no transfection*) or those transfected with ^{Q209L}Gαq or siRNA against Gαq. The average I_{ami} for the control group is 5.94 ± 0.68 μA/cm^2 (n = 6). *C* and *D* (*upper panel*), agarose gel stained with ethidium bromide shows amplified products from RT-PCR from untransfected FRT cells (control), FRT cells transfected with scrambled siRNA, ^{Q209L}Gα11 (*C*), ^{Q205L}Gα14 (*D*), or siRNA directed against Gα11 (*C*) or Gα14 (*D*). Scrambled siRNA and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were used as a negative control for specific siRNAs and loading control, respectively. *C&D* (*lower panel*), Quantitative RT-PCR analysis of mRNA levels of Gα11 in pcDNA3.1 (control), scrambled siRNA, ^{Q209}Gα11 or siRNA against Gα11 transfected into FRT cells (*C*), and Gα14 in pcDNA3.1 (control), scrambled siRNA ^{Q205}Gα14 or siRNA against Gα14 transfected into FRT cells (*D*). Reaction efficiencies were calculated from the slope of the amplification plots for Gα11 (0.981), Gα14 (0.921), and GAPDH (0.958). The results were normalized with GAPDH (n = 3–4). *E*, $I_{ami\ (normalized)}$ of FRT cells described in *C* and *D*. The average I_{ami} for the control group is 5.21 ± 1.35 μA/cm^2 (n = 6). *, **, and *** indicate $p < 0.05$, 0.01, and 0.001, respectively. The number of experiments is shown in *parentheses*.

uncleaved ENaC (Fig. 3*D*) suggests that overexpression of ^{Q209L}Gαq does not significantly change proteolysis of α-ENaC, hence, it is unlikely that inhibition of ENaC by Gαq observed in this study is due to the effect of Gαq on proteolysis of the channel.

The Effect of Gαq on ENaC Is Nedd4-2-independent—Several regulators of ENaC, including the hormones aldosterone, insulin, and vasopressin, and non-hormone regulators, such as intracellular Na^+, modulate activity of the channel by regulating the abundance of ENaC expressed at the cell membrane

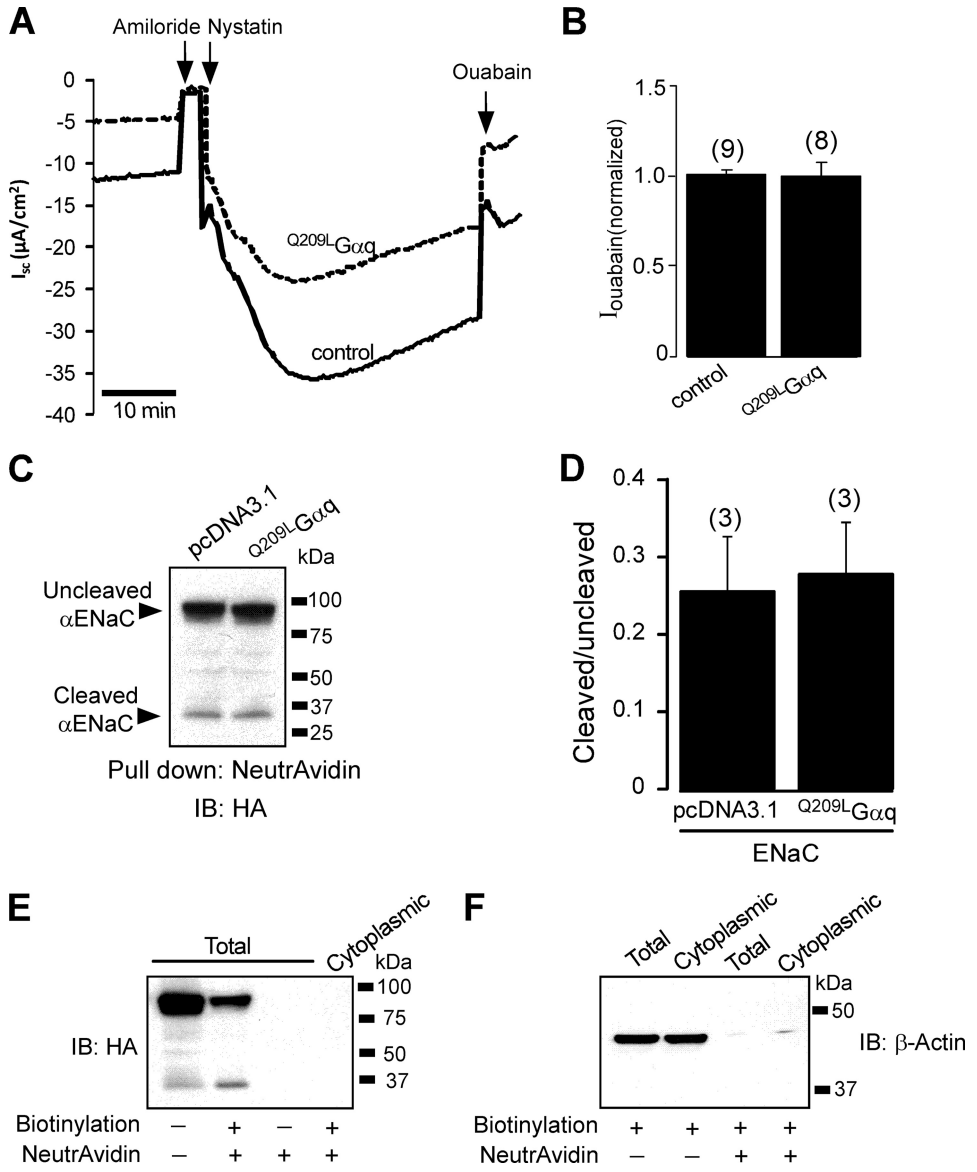

FIGURE 3. **Gαq has no effect on the activity of Na^+/K^+ ATPase or proteolytic cleavage of ENaC.** *A*, short circuit current recording of M1 cell monolayers transfected with ^{Q209L}Gαq or without (control). Amiloride (10 μM) was applied to the apical bath solution to eliminate activity of ENaC before the apical membrane was permeabilized by nystatin (360 μg/ml). After 30 min, ouabain (1 mM) was added to the solution bathing the basolateral membrane. Tracings are representative of at least 8 experiments. *B*, normalized ouabain-sensitive current of experiments in *A*. *C*, immunoblot analysis of ENaC subunits in HEK293T cell transfected with HA-α-V5, β-FLAG and γ-FLAG mENaC. ENaC at the membrane surface was biotinylated, isolated with NeutrAvidin, and immunoblotted with an antibody directed against HA to detect proteolytic fragments of α-ENaC N-terminal. *D*, density of expression of HA-tagged α-ENaC was determined by densitometric analysis and the ratio between the densities of 30 kDa α-ENaC fragment and that of the 95 kDa uncleaved ENaC was determined. *E*, detection of HA-tagged proteins in HEK293T cells transfected with ENaC clones, as in *C*, by immunoblotting with an HA antibody. Total cell proteins from non-biotinylated cells (*lane 1*) and after pull down with NeutrAvidin-agarose (*lane 3*). Total protein (*lane 2*) and cytoplasmic proteins (*lane 4*) from biotinylated cells were pulled down with NeutrAvidin-agarose. *F*, detection of β-actin in total cell lysates (*lane 1*) and in cytoplasmic extracts (*lane 2*) of HEK293T cells transfected with ENaC clones as in *C*. The cytoplasmic extracts were prepared by incubating HEK293T cells for 10 min in a low salt buffer solution containing (in mM) KCl (10), EDTA (1), EGTA (1), dithiothreitol (1), HEPES (50), and 1× protease inhibitor mixture (Roche Applied Science), pH 7.9. Triton X-100 was then added to the cell suspension to a final concentration of 0.5%. The mixture was centrifuged at 6000 rpm at 4 °C for 30 s, and the supernatant was collected as cytoplasmic extract. To demonstrate that there is no biotinylated protein present in the cytoplasmic fraction, total proteins (*lane 3*) and cytoplasmic proteins (*lane 4*) were pulled down with NeutrAvidin-agarose and then detected with an anti-β-actin antibody. *IB*, immunoblot.

(20). The mechanisms by which these regulators control membrane expression of ENaC converge at Nedd4-2 (20), an ubiquitin protein-ligase that triggers ubiquitin-dependent internalization of ENaC. Of particular interest is that cytosolic Na^+ increases Nedd4-2-dependent internalization of ENaC (14) by a mechanism that involves activation of the α-subunit of the G protein Go (13). This suggests that Nedd4-2 may be involved in the Gq/11 protein-mediated signaling pathways that down-regulate ENaC activity. We investigated this possibility by generating mutant ENaC subunits, α_T, β_T-, and γ_T-ENaC, which were truncated at the amino acids Arg-646, Cys-594, and Phe-610, respectively, to remove their C termini, including the proline-rich PY-motifs, which are the binding sites for Nedd4-2. Each of these mutated ENaC subunits was co-tranfected into FRT cells with appropriate wt-ENaC subunits, *i.e.* $\alpha_T\beta\gamma$, $\alpha\beta_T\gamma$, or $\alpha\beta\gamma_T$, or co-transfected *i.e.* $\alpha_T\beta_T\gamma_T$, with or without the

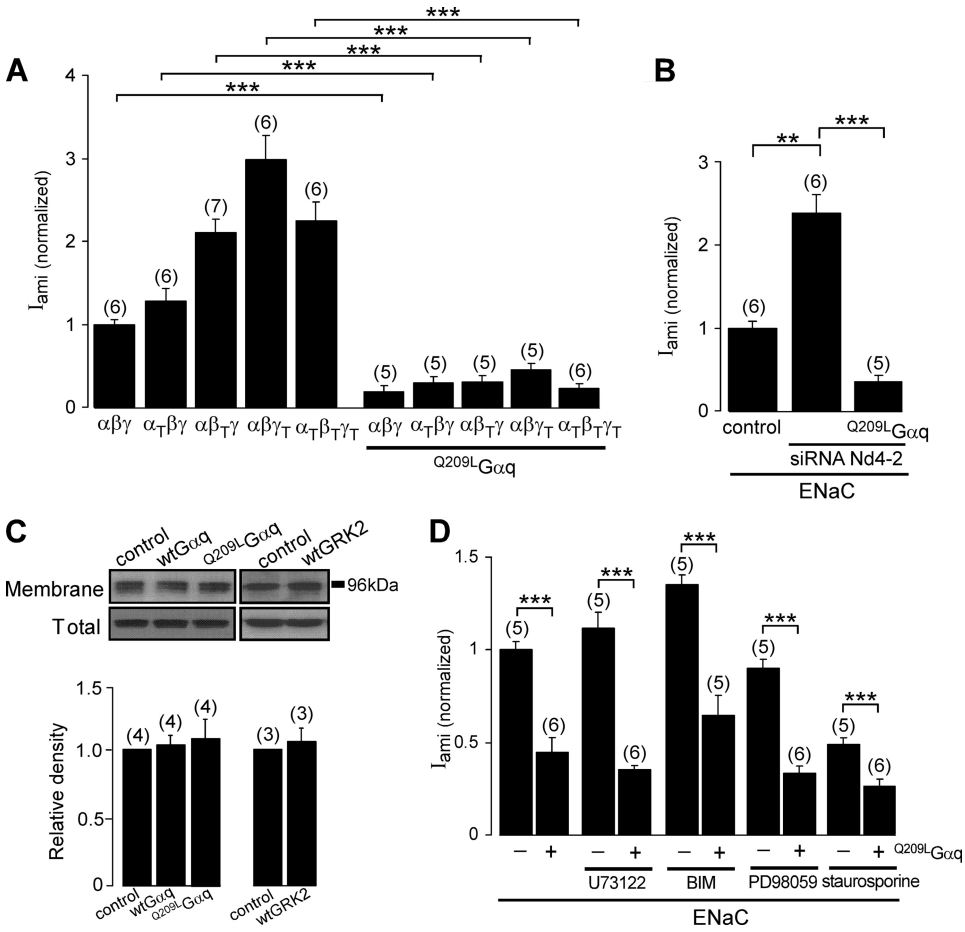

FIGURE 4. **The effect of Gαq on ENaC is not dependent on Nedd4-2 or known common pathways.** *A*, $I_{ami\ (normalized)}$ in FRT cells transfected with wild-type α-, β-, and γ-ENaC (αβγ), wild-type βγ-ENaC with mutant α-ENaC that is truncated at amino acid Pro^{646} ($\alpha_T\beta\gamma$), wild-type αγ-ENaC with mutant β-ENaC that is truncated at amino acid Cys^{594} ($\alpha\beta_T\gamma$), wild-type αβ-ENaC with mutant γ-ENaC that is truncated at amino acid Phe^{610} ($\alpha\beta\gamma_T$) or co-transfected with $\alpha_T\beta_T\gamma_T$ ENaC. The *solid bar* indicates that the cells were co-transfected with ^{Q209L}Gαq. The average I_{ami} for the control group is 6.18 ± 1.42 μA/cm² (n = 6). *B*, $I_{ami\ (normalized)}$ in FRT cells transfected with three subunits of ENaC (*control*) or co-transfected with ENaC and ^{Q209L}Gαq. The cells were co-transfected with siRNA against Nedd4–2 (siRNA Nd4–2) as indicated. The average I_{ami} for the control group is 3.87 ± 0.49 μA/cm² (n = 6). *C*, representative immunoblot analysis for FLAG-tagged membrane protein in HEK293T cells co-transfected with ENaC-FLAG and an empty pcDNA3.1 vector to keep the plasmid concentration the same for all experiments or co-transfected with ENaC-FLAG together with wtGαq, ^{Q209L}Gαq, or wtGRK2. Proteins at the membrane surface of HEK293T cells were biotinylated and pulled down with NeutrAvidin and detected by immunoblotting using anti-FLAG M2 monoclonal antibody. The relative density of the FLAG-tagged membrane protein to that of the FLAG-tagged protein in total cell lysates was determined by densitometric analysis and shown in the *lower panel*. *D*, $I_{ami\ (normalized)}$ in FRT cells transfected with ENaC or co-transfected with ENaC and ^{Q209L}Gq as indicated. *Solid bars* indicate that cells were treated with U73122 (10 μM), BIM (1 μM), PD98059 (20 μM), or staurosporine (100 nM). The average I_{ami} for the control group is 4.25 ± 1.01 μA/cm² (n = 5). ** and *** indicate $p < 0.01$ and 0.001, respectively. The number of experiments is shown in *parentheses*.

constitutively active ^{Q209L}Gαq mutant. Activity of ENaC in these cell monolayers was then determined in Ussing chamber experiments. Consistent with the role of Nedd4-2 in regulating ENaC activity, the normalized amiloride-sensitive current in cells expressing the mutated ENaC subunits, which cannot interact with Nedd4-2, was significantly higher than that of the cells expressing wild-type ENaC subunits (Fig. 4*A*). We further found that co-expression of the ^{Q209L}Gαq mutant was equally as effective in inhibiting the amiloride-sensitive current in cells expressing the C-terminal-truncated mutant of ENaC as in those expressing wtENaC (Fig. 4*A*), which indicates that the inhibition of ENaC activity by Gαq is independent of the presence of the PY-motifs. This implies that it is not mediated by Nedd4-2. To confirm this finding, we inhibited expression of Nedd4-2 in FRT cells by transfecting the cells with siRNA directed against Nedd4-2. As was shown in our earlier study (27), this maneuver significantly increased the amiloride-sensitive current in the transfected cells (Fig. 4*B*). Expression of ^{Q209L}Gαq together with siRNA against Nedd4-2 decreased the normalized amiloride-sensitive current by 73% of that observed in a control group which was not transfected with the siRNA (Fig. 4*B*), hence, we conclude that the inhibitory effect of Gαq on ENaC is independent of Nedd4-2.

To determine whether Gαq inhibits ENaC by reducing membrane expression of the channel, we investigated the effect of overexpression of Gαq on the abundance of ENaC at the cell membrane by co-transfecting HEK293T cells with ENaC and wild-type Gαq or constitutively active ^{Q209L}Gαq mutant. Analysis of the abundance of ENaC present in membrane fractions isolated from these cells revealed that expression of the wtGαq, ^{Q209L}Gαq mutant or wtGRK2 had no effect on membrane expression of ENaC (Fig. 4*C*).

To further investigate the mechanism by which Gαq/11 regulates the activity of ENaC, we used different pharmacological

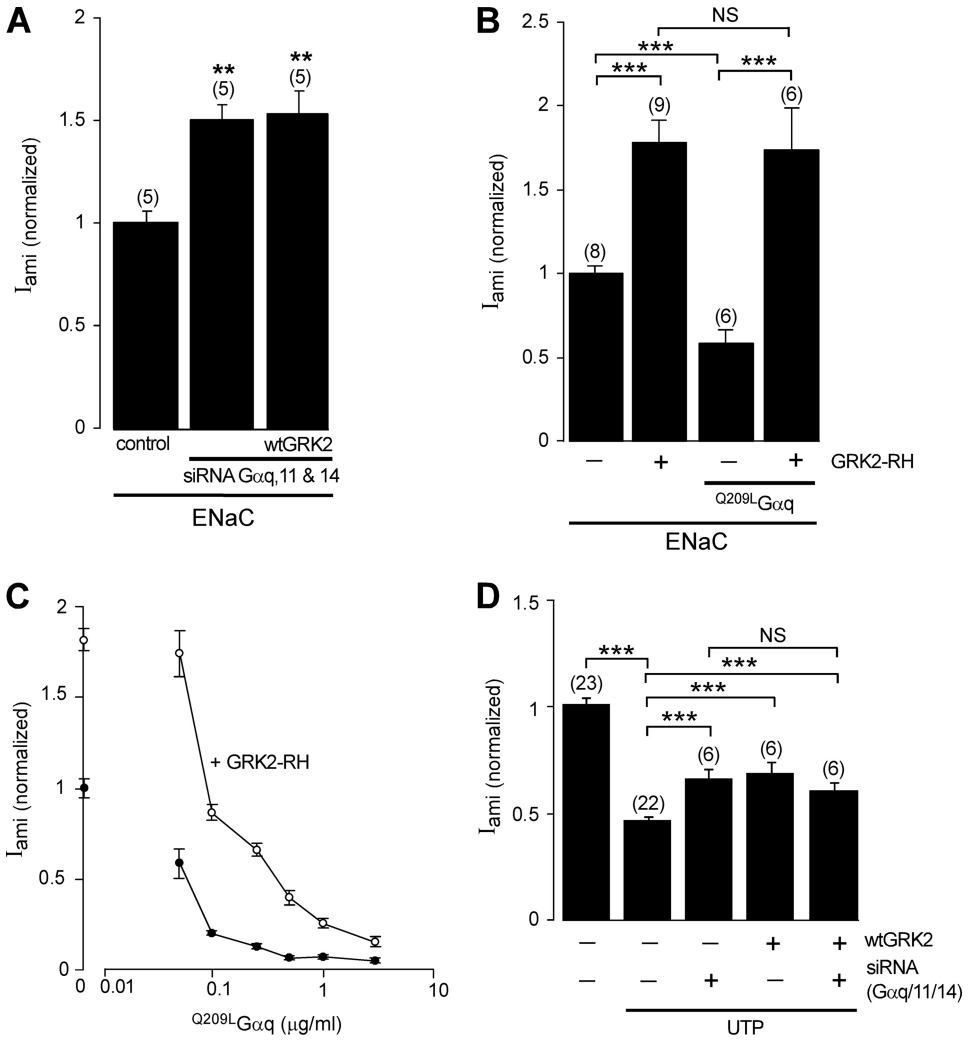

FIGURE 5. **RH domain of GRK2 up-regulates ENaC activity by inhibiting Gαq.** *A*, $I_{ami\ (normalized)}$ in FRT cells transfected with ENaC or co-transfected with ENaC and wtGRK2. The *solid bar* indicates that the cells were co-transfected with siRNA directed against Gαq, Gα11, and Gα14 together. The average I_{ami} for the control group is 14.31 ± 2.27 μA/cm^2 (n = 5). *B*, $I_{ami\ (normalized)}$ in FRT cells co-transfected with ENaC and with (+) or without (−) a plasmid containing the RH domain of GRK2 (GRK2-RH, 0.05 μg/ml). The *solid bar* indicates that the cells were co-transfected with ^{Q209L}Gαq. The average I_{ami} for the control group is 8.81 ± 2.92 μA/cm^2 (n = 8). *C*, $I_{ami\ (normalized)}$ in FRT cells co-transfected with ENaC and various concentrations of ^{Q209L}Gαq (0.05, 0.1, 0.25, 0.5, 1, and 3 μg/ml, *closed circles*) or ^{Q209L}Gαq and GRK2-RH (3 μg/ml, *open circles*) (n = 5–6). *D*, $I_{ami\ (normalized)}$ in M1 cells transfected with (+) and without (−) wtGRK2 and siRNAs directed against Gαq, Gα11, and Gα14. The solid bar indicates that cells were treated with UTP (100 μM). The average I_{ami} for the control group is 4.47 ± 0.47 μA/cm^2 (n = 23). ** and *** indicate $p < 0.01$ and 0.001, respectively. The number of experiments is shown in parentheses. NS indicates no significant difference between groups.

blockers of signaling proteins known to be downstream to Gαq/11, including phospholipase Cβ (PLCβ) inhibitor, U73122, protein kinase C (PKC) inhibitor, bisindolylmaleimide-1 (BIM), and ERK1/2 inhibitor, PD98059, to assess the effect of Gαq on ENaC (Fig. 4*D*). We found that these blockers do not change the inhibitory effect of the constitutively active ^{Q209L}Gαq mutant on the activity of exogenous ENaC expressed in FRT cells (Fig. 4*D*). Neither was the nonspecific kinase inhibitor, staurosporine, able to prevent the effect of ^{Q209L}Gαq on ENaC (Fig. 4*D*).

GRK2 Increases Activity of ENaC by Inhibiting Gαq—Our data suggest that the binding site for Gαq/11 on GRK2 is critical to the non-kinase mediated effect of GRK2 on ENaC. To confirm that GRK2 increases the activity of ENaC by binding Gαq, we suppressed expression of Gαq/11 in FRT cells by co-transfecting the cells with a combination of siRNAs directed against Gαq, Gα11, and Gα14. These cells were then tranfected with ENaC with or without wtGRK2. As shown in Fig. 5*A*, suppressing Gαq, Gα11, and Gα14 significantly increased activity of ENaC. Co-expression of wtGRK2 did not increase the activity of ENaC in the cells further. The lack of effect of GRK2 in cells in which expression of Gαq, Gα11, and Gα14 was knocked down suggests that the stimulatory effect of GRK2 on ENaC may be associated with activity of Gαq/11.

To determine whether GRK2 can overcome the inhibitory effect of Gαq on ENaC, we co-tranfected FRT cells with ENaC and the constitutively active ^{Q209L}Gαq mutant together with a construct containing the RH domain of GRK2 (GRK2-RH) (36). Expression of GRK2-RH significantly increased normalized amiloride-sensitive current in this cell type when compared with that of a control group without GRK2-RH (1.78 ± 0.13, n = 9 *versus* 1.00 ± 0.04, n = 8; $p < 0.001$, Fig. 5*B*). Expression of ^{Q209L}Gαq (0.05 μg/ml) decreased the normalized amiloride-sensitive current to 0.58 ± 0.08 (n = 6; $p < 0.001$, Fig. 5*B*). Co-expression of GRK2-RH restored the activity of ENaC to a

level similar to that observed in another group of cells that were transfected with GRK2-RH in the absence of ^{Q209L}Gαq mutant (Fig. 5*B*). These findings suggest that GRK2-RH can completely overcome the inhibitory effect of Gαq on ENaC.

As mentioned in the Introduction, the RH domain of GRK2 contains a binding site that selectively binds to and inhibits Gαq, Gα11, and Gα14. We envisage that, if binding to GRK2 prevents Gαq from inhibiting ENaC, the ability of GRK2-RH to prevent ENaC inhibition by Gαq should be attenuated at high concentration of Gαq. To investigate this, we co-transfected FRT cells with ENaC with different concentrations of ^{Q209L}Gαq. We found that Gαq inhibits activity of ENaC in a concentration-dependent manner (Fig. 5*C*). Co-expression of GRK2-RH restored activity of ENaC in ^{Q209L}Gαq-transfected cells and shifted the concentration-response curve of ENaC to ^{Q209L}Gαq expression to the right (Fig. 5*C*). When transfected with 0.05 μg/ml ^{Q209L}Gαq plasmid, the normalized amiloride-sensitive current in FRT cells was 0.58 $\pm$ 0.08 ($n = 6$), representing a 42% inhibition compared with that of the control ENaC-only-transfected cells (1.00 $\pm$ 0.04, $n = 8$; $p < 0.001$). Co-expression of GRK2-RH with ^{Q209L}Gαq increased the normalized amiloride-sensitive current to 1.74 $\pm$ 0.25 ($n = 6$), a value that is not significantly different to that observed in cells co-tranfected with GRK2-RH but without ^{Q209L}Gαq (1.78 $\pm$ 0.13, $n = 9$; $p > 0.05$). On the other hand, expression of ^{Q209L}Gαq at a concentration of 0.3 μg/ml reduced the normalized amiloride-sensitive current to 0.05 $\pm$ 0.01 ($n = 6$), an inhibition of 95% when compared with that of the control untransfected cells. Under these conditions, GRK2-RH failed to fully restore activity of ENaC, only increasing the normalized amiloride-sensitive current to 0.15 $\pm$ 0.02 ($n = 6$). The reciprocal relationship between ^{Q209L}Gαq concentration and the ability of GRK2 to restore activity of ENaC is consistent with the effect of GRK2 on ENaC being dependent upon the ability of GRK2 to directly bind to, and prevent, Gαq from inhibiting the channel, which becomes less effective in the presence of higher concentrations of activated Gαq.

It has been suggested that $P2Y_2$ purinoceptors are coupled with Gq/11 (37) and that activation of the receptors inhibits activity of ENaC (38, 39). We found that siRNAs directed against Gαq, Gα11, and Gα14 attenuated the inhibitory effect of the $P2Y_2$ receptor agonist, uridine-tri-phosphate (UTP), on the activity of ENaC (Fig. 5*D*), suggesting that the mechanism by which $P2Y_2$ receptor activation inhibits the activity of ENaC is mediated, in part, via Gq/11. Since expression of wtGRK2 blunts the effect of UTP on ENaC in a similar manner to the effect of the siRNAs against Gq/11/14 and the effect of GRK2 and the siRNAs are not additive (Fig. 5*D*), it is most likely that GRK2 exerts its effect on UTP signaling by inhibiting Gq/11, hence disrupting part of the signaling pathway activated during $P2Y_2$ receptor activation.

DISCUSSION

The present study has provided evidence that GRK2 functions as an endogenous activator of ENaC in epithelia. Inhibition of GRK2 by siRNA significantly decreased activity of ENaC in cells in which the channel is endogenously expressed. Our data also elucidate the motifs in GRK2, which are responsible for this function. The ability of a kinase-inactivated GRK2, ^{K220R}GRK2, to increase the activity of ENaC similarly to that of the wild-type GRK2, indicates that this effect of GRK2 is independent of its intrinsic kinase activity. The effect of GRK2 is, however, mediated by its RH domain, because the ΔRH-GRK2 mutant which lacks this domain was ineffective in regulating activity of ENaC. An investigation using a construct containing only the RH domain of GRK2 further revealed that the presence of the RH domain alone increased activity of the channel.

The phosphorylation-independent effect mediated by the RH domain of GRK2 revealed in the current study identifies an additional stratum of regulation provided by this kinase to control epithelial Na^+ absorption. Unlike its phosphorylation-dependent mechanism, which impinges upon Nedd4-2 and acts downstream in the Gαo-mediated signaling system that regulates ENaC (13, 22, 40), the RH domain of GRK2 mediates its effect by inhibiting the effect of Gαq on the channel. The lack of kinase effect of GRK2 on activity of ENaC reported in this study may reflect the absence of the Nedd4-2-dependent GRK2 signaling system that regulates ENaC in cultured epithelia.

The RH domain of GRK2 has been implicated in the regulation of several G protein signaling events (3) including insulin-stimulated glucose transporter 4 translocation in adipocytes (26) and phosphoinositide hydrolysis during activation of several GPCRs, including the endothelin receptor (41), metabotropic glutamate receptor (42), parathyroid hormone receptor (43), angiotensin receptor (44), and thromboxane A_2 receptor (7). Together, these findings suggest that the RH domain plays a central role in mediating the physiological effects of GRK2. In the present study, the failure of the ^{D110A}GRK2 mutant, in which an amino acid in its RH domain was mutated to prevent binding of GRK2 to Gαq/11 (28), to regulate the basal activity of ENaC suggests that direct binding of GRK2 to Gαq/11 is essential for the mechanism by which GRK2 activates ENaC in this experimental system. The reciprocal relationship between the concentration of Gαq and the ability of the RH domain of GRK2 to prevent the ^{Q209L}Gαq mutant from inhibiting ENaC further suggests that the RH domain of GRK2 may act as an effector antagonist. This phosphorylation-independent effect of GRK2 on Gα is likely to act in concert with its intrinsic catalytic activity to maximize the negative effect of GRK2 on G protein signaling that controls activity of ENaC.

One important contribution of this study is that it is the first to demonstrate the negative regulatory effect of Gαq/11 on the channel. Data from our siRNA studies suggest that, under basal conditions, there is a tonic effect of endogenous Gαq/11 that suppresses activity of ENaC in epithelia. Similar activation of ENaC was observed with siRNA directed against Gαq, Gα11 or Gα14. These Gα subunits share over 80% homology in their amino acid sequences (45), hence their ability to inhibit the channel might reflect a promiscuous capacity to activate the same downstream signaling pathways, one of which inhibits ENaC. Gq/11-coupled GPCRs are known to regulate several cellular events in epithelial cells, including intracellular Ca^{2+} mobilization (46), mucin secretion (47, 48), and anion secretion (49). Our data suggest that, Gq/11 plays a small, but significant, part in the $P2Y_2$ receptor-mediated signaling mechanism that inhibits activity of ENaC. The fact that

GRK2 rescues part of ENaC activity during $P2Y_2$ receptor activation, most likely by inhibiting Gq/11, further emphasizes the physiological significance of GRK2 in the regulation of Na^+ transport in epithelia.

The nature of these signaling pathways remains unclear, as our data exclude the pathways activated by Gαq/11 which are known to inhibit ENaC. For example, activation of phospholipase Cβ by Gαq/11 can initiate hydrolysis of inositol lipids, especially phosphatidylinositol 4,5-bisphosphate (PIP_2), (45) leading to inhibition of ENaC (38, 50). Our finding that the inhibitor of PLC, U73122, does not prevent inhibition of ENaC by the ^{Q209L}Gαq mutant makes it unlikely that U73122-sensitive PLCβ isoforms are involved in the mechanism by which Gαq inhibits ENaC. In this regard, a role for PLC isoforms that are less sensitive to U73122 (51) cannot be excluded. Similarly, it is unlikely that Gαq exerts its effect on ENaC via PKC, ERK1/2 or some other staurosporine-sensitive protein kinase, as expression of Gαq is effective in inhibiting activity of ENaC in cells pretreated with pharmacological inhibitors of these kinases. It has also been suggested that expression of the constitutively active ^{Q209L}Gαq inhibits phosphatidylinositol 3-kinase (PI3K), a known activator of ENaC (52), by a mechanism that is independent of PLC activation (53). The effect of Gq on PI3K, however, cannot explain the negative effect of Gαq on ENaC in our study since the inhibitory effect of Gαq could still be observed in cells over-expressing a dominant-negative mutant of PI3K, Δp85 PI3K (54) (data not shown). Finally, Chen *et al.* (55) suggested that the inhibitory effect of Gαq on the activity of the two-pore-domain K^+ channel may be due to a direct binding of the Gα to the channel. This effect of Gαq is independent of PLC or PIP_2. Our co-immunoprecipitation assay, however, has failed to detect any interaction between Gαq and ENaC subunits (data not shown), hence, a direct inhibitory effect of Gαq on the activity of ENaC cannot be confirmed in this experimental system.

We have shown previously that the activity of ENaC is regulated by α- of Go and Gi2 (13, 56). Gαo is a component of the Na^+ feedback signaling pathway that inhibits activity of ENaC in the presence of high cytosolic Na^+ concentration (13, 15). This effect of Go is mediated by ubiquitin protein ligases of the Nedd4 family (14, 18, 57), which increase internalization of membrane ENaC, hence decreasing membrane expression and activity of the channel (58). Gαi2 is a component of the anion feedback pathway, which is activated by high cytosolic Cl^- concentrations (13), however, unlike the effect of Gαo, its inhibitory effect on ENaC does not involve Nedd4 family proteins (14). Our data indicate that the effect of Gαq on ENaC does not depend upon the presence of the PY-motifs at the C-terminal of ENaC subunits, which are the binding sites of Nedd4 proteins, nor is it attenuated in cells in which siRNA had been used to knock down Nedd4-2 activity. Moreover, overexpression of ^{Q209L}Gαq has no effect on membrane expression of ENaC. Hence, like Gαi2, the mechanism by which Gαq inhibits activity of ENaC does not involve Nedd4 proteins. At present, the signaling pathway mediated by Gαq to regulate activity of ENaC remains elusive.

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