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

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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 \(\alpha\)-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 \((K220R)\), but not a GRK2 mutant that lacks the C-terminal RH domain \((\Delta RH)\) or a GRK2 mutant that cannot interact with Gq/11/14 \((D^{110A})\), increase activity of ENaC. GRK2 up-regulates the basal activity of the channel as a consequence of its RH domain binding the \(\alpha\)-subunits of Gq/11. We further found that expression of constitutively active Gq/11 mutants significantly inhibits its activity of ENaC. Conversely, co-expression of siRNA against Gq/11 increases ENaC activity. The effect of Gaq 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 \(\alpha\)-subunits regulate the activity ENaC.

The \(\beta\)-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 binds a binding site that selectively interacts with the \(\alpha\)-subunits \((Go)\) of the trimeric G proteins Gq, G11, and G14, but excludes Go of other Gq/11 family members and those of Gi, Go, or Gs (3, 5–7). Binding of GRK2 to Gq/11 effectively terminates transduction of the Go 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 \(\alpha\)-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 \(\beta\)-subunit of ENaC (17) and Nedd4 and Nedd4-2 (22), this phosphorylation-dependent effect of GRK2 on ENaC might...
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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, K220RGRK2, D110A-GRK2, RH domain of GRK2, wtGαq, and Q209LG-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). Q209LG-Gα11, Q209LG-Gα14, and Q212LG-Gα15/16 in pcDNA3 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 Pro646stop α-ENaC, Cys594stop β-ENaC or Phe610stop 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 overnight with anti-GRK2 monoclonal antibody, anti-Gq, or anti-HA monoclonal antibody (Cell Signaling Technology), anti-FLAG M2 monoclonal antibody (Sigma), or anti-HA monoclonal antibody (Cell Signaling Technology). The media for M1 cells contained 10% fetal bovine serum whereas the media for M1 cells contained 10% fetal bovine serum and 5% fetal bovine serum.

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.

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 AAG GCC AAT GC-3’); reverse, 5′-GAA GAT GAT GTT CTC CAG GTC-3’), Gα14 (forward, 5′-CAG AAG GAA AAT GCC CAG-3’); reverse, 5′-CCT GGT CAT ATT CAC TCA GAG-3’), and GAPDH (forward, 5′-TGG CCT TTC GTG TTT 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.

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-Gq polyclonal antibody, or anti-β-actin monoclonal antibody (Santa Cruz Biotechnology). The monolayers became confluent, normally within 2–3 days after transfection, the Millicell-PCF permeable filter supports (Millipore, catalogue number PIP01250). One day after seeding, FRT cells were co-transfected with cDNA of α-, β-, and γ-ENaC-FLAG (0.7 μg/ml each). When appropriate, FRT and M1 cells were transfected with cDNA of wtGRK2, mutants of GRK2, mutants of Gqα, Gα11 or Gα14 (3 μg/ml), siRNA against GRK2, siRNA against Nedd4-2 or siRNA against Gqα, Gα11, or Gα14 (0.5 μg/ml). siRNAs against GRK2 (5′-GGA CAC AAA AGG AATCAA GTT-3′) and Gαq (5′-GGC TCA TGC ACA ATT GTT-3′) were obtained from Ambion and siRNAs against Gα11 (5′-CAC AAC TGC CAT CCT GCA GTA-3′), Gα14 (5′-TCT GGT GCT CTT AGT GAA TAT-3′) and Nedd4-2 (5′-AAC CAC AAC 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. The epithelial 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 ($I_{\text{ami}}$) was determined as the change in current following the addition of amiloride (10 μM) to the apical bathing solution. Because of large variation of $I_{\text{ami}}$ 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 ($I_{\text{ami(normalized)}}$). The mean value of $I_{\text{ami}}$ 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.

Quantification 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 eradicare 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 endogenously express ENaC by transfecting the cells with a plasmid containing wild-type GRK2 (wtGRK2) (Fig. 1A, 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 over-expressing a kinase-dead GRK2 mutant (K220RGRK2) (Fig. 1A), in which the invariant lysine residue 220 in the protein kinase

![Figure 1](image-url)

**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 (K220RGRK2) or an RH domain deletion mutant of GRK2 (∆RH-GRK2), showing the response to 10 μM amiloride (solid bar). A lower panel, immunoblot analysis of expression of the wtGRK2, K220RGRK2 and ∆RH-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 ($I_{\text{ami(normalized)}}$) in untransfected M1 cells (control) or cells transfected with wtGRK2, K220RGRK2 or ∆RH-GRK2 as in A. The average $I_{\text{ami}}$ for the control group is 20.63 ± 2.37 μA/cm² (n = 6). C, immunoblot analysis (upper panel) and $I_{\text{ami(normalized)}}$ (lower panel) in FR7 cells transfected with three subunits of ENaC (control) or co-transfected with ENaC and with wild-type GRK2 (wtGRK2), K220RGRK2, or ∆RH-GRK2, showing the response to 10 μM amiloride (solid bar). A lower panel, immunoblot analysis of expression of the wtGRK2, K220RGRK2 and ∆RH-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 ($I_{\text{ami(normalized)}}$) in untransfected M1 cells (control) or cells transfected with wtGRK2, K220RGRK2 or ∆RH-GRK2 as in A. The average $I_{\text{ami}}$ for the control group is 20.63 ± 2.37 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.
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catalytic domain was converted to an arginine to terminate kinase activity (25). We found that activity of ENaC in cells expressing the K220GRK2 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 K220GRK2 mutant on ENaC was not significantly different from that of the wtGRK2 (Fig. 1B). 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. 1B). 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. 1C). 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. 1C). 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 K220GRK2 mutant (1.58 ± 0.09, n = 6) was significantly higher than that of the control group (p < 0.001). Expression of the K220GRK2 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. 1C). 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. 1C). Under these conditions, the normalized amiloride-sensitive current (0.89 ± 0.09, n = 4) observed in the co-transfected 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 (D110AGRK2), 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 Goq/11/14 (28, 29). We found that, the D110AGRK2 mutant failed to alter activity of ENaC (Fig. 1C), supporting the conclusion that the observed effect of GRK2 on ENaC may be due to GRK2 preventing Goq from inhibiting the activity of ENaC.

Gq/11 Family Proteins Inhibit Activity of ENaC—We then investigated the effect of Goq/11 on ENaC activity by suppressing expression of Goq using an siRNA in FRT cells co-transfected with ENaC (Fig. 2A). We found that normalized amiloride-sensitive current in Goq 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 Goq mutant, Q209L-Goq (30, 31), which decreased the normalized amiloride-sensitive current in FRT cells (0.15 ± 0.05, n = 8; p < 0.001; Fig. 2A), suggesting that Goq 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-Goq mutant abolished activity of ENaC and the siRNA directed against Goq significantly increased activity of the channel (Fig. 2B).

To determine whether ENaC is also regulated by the α-subunits of Go11 and Go14, which are known to interact with GRK2, we expressed constitutively active Q209L-Go11 and Q209L-Go14 mutants in FRT cells. Expression of Q209L-Go11 and Q209L-Go14 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-Go11 and Q205L-Go14 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-Go11 and Q205L-Go14 mutants inhibited the amiloride-sensitive Na+ current (Fig. 2E). Conversely, siRNAs directed against Go11 and Go14 attenuated expression of Go11 and Go14 (Fig. 2, C and E) and significantly increased activity of ENaC (Fig. 2E). In addition, expression of the constitutively active Q212A-Go15/16 mutant also inhibited the amiloride-sensitive Na+ current (data not shown). Hence, the Go of all of the Gq/11 family act as negative-regulators of ENaC activity.

To eliminate a possibility that the inhibitory effect of Goq on ENaC activity may be due to Goq 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 Goq on the ouabain-sensitive current which represents activity of the Na+/K+-ATPase. We found that expression of Q209L-Goq 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 Goq 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-Goq. 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. 3C with control biotinylation in Fig. 3E). No biotinylated proteins were detected in the cytoplasmic fraction (Fig. 3F). A ratio between the density of the 30 kDa α-ENaC N-terminal fragment and that of 95 kDa
uncleaved ENaC (Fig. 3D) suggests that overexpression of Q209LG does not significantly change proteolysis of α-ENaC, hence, it is unlikely that inhibition of ENaC by Gaq observed in this study is due to the effect of Gaq on proteolysis of the channel.

The Effect of Gaq 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.
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+/H+ 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, α2γ, β2γ-, and γ1-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-transfected into FRT cells with appropriate wt-ENaC subunits, i.e. α2γγ2βγ, α2γγ2γ, or αβγγ, or co-transfected i.e. α1β1γ1, with or without the

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FIGURE 3. Gq 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-Gqα 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 1X 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.
constitutively active Q209LG-Gaql 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. 4A). We further found that co-expression of the Q209LG-Gaql 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. 4A), which indicates that the inhibition of ENaC activity by Gaql 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. 4B). Expression of Q209LG-Gaql 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. 4B), hence, we conclude that the inhibitory effect of Gaql on ENaC is independent of Nedd4-2.

To determine whether Gaql inhibits ENaC by reducing membrane expression of the channel, we investigated the effect of overexpression of Gaql on the abundance of ENaC at the cell membrane by co-transfecting HEK293T cells with ENaC and wild-type Gaql or constitutively active Q209LG-Gaql mutant. Analysis of the abundance of ENaC present in membrane fractions isolated from these cells revealed that expression of the wtGaql, Q209LG-Gaql mutant or wtGRK2 had no effect on membrane expression of ENaC (Fig. 4C).

To further investigate the mechanism by which Gaql/11 regulates the activity of ENaC, we used different pharmacological
blockers of signaling proteins known to be downstream to Gq/11, including phospholipase C (PLC) inhibitor, U73122, protein kinase C (PKC) inhibitor, bisindolylmaleimide-1 (BIM), and ERK1/2 inhibitor, PD98059, to assess the effect of Gq on ENaC (Fig. 4D). We found that these blockers do not change the inhibitory effect of the constitutively active Q209L-Gq on ENaC (Fig. 4D). Neither was the nonspecific kinase inhibitor, staurosporine, able to prevent the effect of Q209L-Gq on ENaC (Fig. 4D).

**GRK2 Increases Activity of ENaC by Inhibiting Gq**—Our data suggest that the binding site for Gq/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 Gq, we suppressed expression of Gq/11 in FRT cells by co-transfecting the cells with a combination of siRNAs directed against Gq, Ga11, and Ga14. These cells were then transfected with ENaC with or without wtGRK2. As shown in Fig. 5A, suppressing Gq, Ga11, and Ga14 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 Gq, Ga11, and Ga14 was knocked down suggests that the stimulatory effect of GRK2 on ENaC may be associated with activity of Gq/11.

To determine whether GRK2 can overcome the inhibitory effect of Gq on ENaC, we co-transfected FRT cells with ENaC and the constitutively active Q209L-Gq 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. 5B). Expression of Q209L-Gq (0.05 μg/ml) decreased the normalized amiloride-sensitive current to 0.58 ± 0.08 (n = 6; p < 0.001, Fig. 5B). 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 Q209LG Qaq mutant (Fig. 5B). These findings suggest that GRK2-RH can completely overcome the inhibitory effect of Qaq on ENaC.

As mentioned in the Introduction, the RH domain of GRK2 contains a binding site that selectively binds to and inhibits Gaq, Ga11, and Ga14. We envisage that, if binding to GRK2 prevents Qaq from inhibiting ENaC, the ability of GRK2-RH to prevent ENaC inhibition by Qaq should be attenuated at high concentration of Qaq. To investigate this, we co-transfected FRT cells with ENaC with different concentrations of Q209LG Qaq. We found that Qaq inhibits activity of ENaC in a concentration-dependent manner (Fig. 5C). Co-expression of GRK2-RH restored activity of ENaC in Q209LG Qaq-transfected cells and shifted the concentration-response curve of ENaC to Q209LG Qaq expression to the right (Fig. 5C). When transfected with 0.05 µg/ml Q209LG Qaq plasmid, the normalized amiloride-sensitive current in FRT cells was 0.58 ± 0.08 (n = 6), representing a 42% inhibition compared with that of the control ENaC-only-transfected cells (1.00 ± 0.04, n = 8; p < 0.001).

Co-expression of GRK2-RH with Q209LG Qaq increased the normalized amiloride-sensitive current to 1.74 ± 0.25 (n = 6), a value that is not significantly different to that observed in cells co-transfected with GRK2-RH but without Q209LG Qaq (1.78 ± 0.13, n = 9; p > 0.05). On the other hand, expression of Q209LG Qaq at a concentration of 0.3 µg/ml reduced the normalized amiloride-sensitive current to 0.05 ± 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 ± 0.02 (n = 6). The reciprocal relationship between Q209LG Qaq 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, Qaq from inhibiting the channel, which becomes less effective in the presence of higher concentrations of activated Qaq.

It has been suggested that P2Y2 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 Qaq, Ga11, and Ga14 attenuated the inhibitory effect of the P2Y2 receptor agonist, uridine-tri-phosphate (UTP), on the activity of ENaC (Fig. 5D), suggesting that the mechanism by which P2Y2 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. 5D), 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 P2Y2 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, k220AGRK2, 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 ARH-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 present 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 Gao-mediated signaling system that regulates ENaC (13, 22, 40), the RH domain of GRK2 mediates its effect by inhibiting the effect of Qaq 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 A2 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 D110AGRK2 mutant, in which an amino acid in its RH domain was mutated to prevent direct binding of GRK2 to Qaq/11 (28), to regulate the basal activity of ENaC suggests that direct binding of GRK2 to Qaq/11 is essential for the mechanism by which GRK2 activates ENaC in this experimental system. The reciprocal relationship between the concentration of Qaq and the ability of the RH domain of GRK2 to prevent the Q209LG Qaq 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 Gq 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 Qaq/11 on the channel. Data from our siRNA studies suggest that, under basal conditions, there is a tonic effect of endogenous Qaq/11 that suppresses activity of ENaC in epithelia. Similar activation of ENaC was observed with siRNA directed against Qaq, Ga11 or Ga14. These Ga 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 Ca2+ mobilization (46), mucin secretion (47, 48), and anion secretion (49). Together our data suggest that, Gq/11 plays a small, but significant, part in the P2Y2 receptor-mediated signaling mechanism that inhibits activity of ENaC. The fact that
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GRK2 rescues part of ENaC activity during P2Y2 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 Goaq/11 which are known to inhibit ENaC. For example, activation of phospholipase Cβ by Goaq/11 can initiate hydrolysis of inositol lipids, especially phosphatidylinositol 4,5-bisphosphate (PIP2), leading to inhibition of ENaC (38, 50). Here, we show that the inhibitor of PLC, U73122, does not prevent inhibition of ENaC by the Q209G mutant makes it unlikely that U73122-sensitive PLCβ isoforms are involved in the mechanism by which Goaq 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 Goaq exerts its effect on ENaC via PKC, ERK1/2 or some other staurosporine-sensitive protein kinase, as expression of Goaq 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 Q209G-Goaq inhibits phosphatidylinositol 3-kinase (PI3K), a known activator of ENaC (52), by a mechanism that is independent of PLC activation (53). The effect of Goaq on PI3K, however, cannot explain the negative effect of Goaq on ENaC in our study since the inhibitory effect of Goaq 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 Goaq on the activity of the two-pore-domain K+ channel may be due to a direct binding of the Go to the channel. This effect of Goaq is independent of PLC or PIP2. Our co-immunoprecipitation assay, however, has failed to detect any interaction between Goaq and ENaC subunits (data not shown), hence, a direct inhibitory effect of Goaq on the activity of ENaC cannot be confirmed in this experimental system.

We have shown previously that the activity of ENaC is regulated by α1 of Goaq and Gi2 (13, 56). Goa 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 Goa 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). Gi2 is a component of the anion feedback pathway, which is activated by high cytosolic Cl− concentrations (13), however, unlike the effect of Goaq, its inhibitory effect on ENaC does not involve Nedd4 family proteins (14). Our data indicate that the effect of Goaq 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 Q209G-Goaq has no effect on membrane expression of ENaC. Hence, like Goa2, the mechanism by which Goaq inhibits activity of ENaC does not involve Nedd4 proteins. At present, the signaling pathway mediated by Goaq to regulate activity of ENaC remains elusive.

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