Ga\textsubscript{12} Differentially Regulates Na\textsuperscript{+}-H\textsuperscript{+} Exchanger Isoforms*

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Activation of several GTPases stimulates Na\textsuperscript{+}-H\textsuperscript{+} exchange, resulting in an increased efflux of intracellular H\textsuperscript{+}. These GTPases include \(a\) subunits of the heterotrimeric G proteins Ga and G13, as well as the low molecular weight GTP-binding proteins Ras, Cdc42, and Rho (Hooley, R., Yu, C.-Y., Simon, M., and Barber, D. L. (1996) J. Biol. Chem. 271, 6152–6158). GTPases coupled to the inhibition of Na\textsuperscript{+}-H\textsuperscript{+} exchange, however, have not been identified. Several neurotransmitters, including somatostatin and dopamine, inhibit Na\textsuperscript{+}-H\textsuperscript{+} exchange through a guanine-nucleotide-dependent mechanism, suggesting the involvement of a GTPase. In this study we determined that mutationally activated of the \(a\) subunit of G12 inhibits the ubiquitously expressed Na\textsuperscript{+}-H\textsuperscript{+} exchanger isoform, NHE1. Transient expression of mutationally activated Ga12 inhibited serum- and Ga13-stimulated NHE1 activity in HEK293 cells and CCL39 fibroblasts. In addition, NHE-deficient AP1 cells stably expressing specific NHE isoforms, mutationally activated Ga12 inhibited NHE1 activity but stimulated activities of the Na\textsuperscript{+}-H\textsuperscript{+} exchanger (NHE) isoforms NHE2 and NHE3. In contrast, mutationally activated Ga13, another member of the Ga12/13 family, stimulated all three NHE isoforms. Although previous studies have identified a parallel action of Ga12 and Ga13 in regulating MAP (mitogen-activated protein) kinases and cell growth, these GTPases have opposing effects on NHE1 activity.

Na\textsuperscript{+}-H\textsuperscript{+} exchangers (NHEs)\textsuperscript{3} comprise a family of electro-neutral countertransport proteins participating in intracellular pH\textsuperscript{5} homeostasis, cell volume regulation, and the transepithelial absorption of Na\textsuperscript{+} (1). At least five mammalian exchanger isoforms, NHE1 to NHE5, have been cloned and characterized (2–7). Although these isoforms have a similar structure, consisting of 10–12 putative transmembrane helixes and a long cytoplasmic carboxyl domain, they are different in their tissue and subcellular distribution, transport kinetics, and response to external stimuli (8). NHE1, the only ubiquitously expressed NHE isoform and the most well studied, functions primarily in regulating pH\textsuperscript{5} and cell volume (1, 9). Increases in NHE1 activity are associated with increased cell proliferation (10, 11), differentiation (12), and neoplastic transformation (13–15). The NHE2 and NHE3 isoforms are localized primarily in the apical membrane of epithelia in the intestine and the renal proximal tubule (3, 5, 6, 10, 11). NHE2 has been suggested to function in volume regulation (16), whereas NHE3 functions primarily in transepithelial Na\textsuperscript{+} absorption (17). NHE4 is found in rat stomach and intestine, and the newly identified NHE5 is present primarily in brain, spleen, and testes. Their functional properties are largely undefined (6, 7).

Activities of the NHE isoforms are regulated by growth factors acting at receptor tyrosine kinases and by hormones and neurotransmitters acting at seven-transmembrane receptors (1, 18). The signaling mechanisms mediating regulation of NHE activities, however, have not been clearly defined. Activation of NHE1 by hormone and growth factor receptors may be mediated by GTPases previously shown to stimulate NHE1, which include Ga\textsubscript{i} (19–21), Ga13 (19–21), and Ha-Ras (13–15). The signaling pathways acting downstream of these GTPases to stimulate NHE1 are distinctly different. Ga\textsubscript{i} acts through a protein kinase C-dependent mechanism (20), whereas Ras acts through a Raf-1- and mitogen-activated protein kinase kinase (MEK)-dependent mechanism (22) and Ga13 through a MEK kinase (MEKK1)-dependent mechanism (22). Our laboratory recently determined that two low molecular weight GTPases of the Rho family, Cdc42 and RhoA, also mediate Ga13 activation of NHE1 (22). Hence, a network of heterotrimeric G protein \(a\) subunits and low molecular weight GTPases couple to the stimulation of NHE1 activity.

We previously determined that the neuropeptide somatostatin inhibits NHE activity through a GTP-dependent mechanism (23, 24). Inhibition of NHE activity is insensitive to pertussis toxin, suggesting it is not mediated by the Go\textsubscript{i} and the Go\textsubscript{q} family of pertussis toxin-sensitive GTPases previously determined to couple to somatostatin receptors (25, 26). Thus there appears to be a role for an undetermined GTPase in mediating the inhibition of NHEs by somatostatin. Our laboratory (19) and others (20, 21) previously identified GTPases coupled to the stimulation of NHE activity by expressing their mutationally activated forms in cells. Mutational substitution at either of two conserved amino acids in Go subunits inhibits the intrinsic GTPase activity of these proteins, resulting in their constitutive binding of GTP and thereby their constitutive activation. Using a similar approach in the current study, we found that mutationally activated Ga12 regulated the activity of three NHE isoforms. Ga12 belongs to the more recently identified pertussis toxin-insensitive G protein subfamily Ga12/13. Although expression of Ga12Q229L was found previously to stimulate Na\textsuperscript{+}-H\textsuperscript{+} exchange in COS-7 cells (20), we have now determined that activated Ga12 specifically inhibits NHE1, but stimulates NHE2 and NHE3.
EXPERIMENTAL PROCEDURES

DNA Constructs and Cell Transfection—Ga12-wt and Ga13-wt were gifts from Melvin I. Simon of the California Institute of Technology. Mutationally active forms of α12 (α12Q229L), α13 (α13Q229L), α11 (α11Q226L), α12 (α12Q229L), ω10 (ω10Q250L), and α12 (ω12Q229L) were constructed as described (19). Wild type and mutationaly active α subunits were transiently expressed in cells by using the LipofectAMINE reagent (Life Technologies, Inc.). Transfection efficiency was routinely 30–35% for CCL39 cells and 60–65% for COS-7 cells, as determined by β-galactosidase staining. HEK293 and AP1 cells, as well as AP1 cells stably expressing NHE1, NHE3, and NHE3 (11), were maintained in minimum Eagle's medium supplemented with 10% fetal bovine serum. COS-7 and CCL39 cells were maintained in Dulbecco's modified Eagle's medium (high glucose) supplemented with 10 or 5% fetal bovine serum, respectively.

Intracellular pH Measurements—18 h after transfection, cells were reseeded onto glass coverslips and maintained in the absence or presence of 5–10% serum for an additional 18 h. NHE activity was determined in a nominally HCO₃⁻-free HEPES-buffered medium containing 145 mM NaCl, 5 mM KCl, 1 mM MgCl₂, 1.8 mM CaCl₂, 1 mM KH₂PO₄, 10 mM glucose, and 30 mM HEPES, titrated to a pH of 7.4. Cells were loaded with 1 μM of the acetoxymethyl ester derivative of the pH-sensitive dye 2,7-bis(carboxyethyl)-5(6)-carboxyfluorescein (BCECF; Molecular Probes) for 15 min at 37 °C without CO₂. Coverslips were transferred to a perfusion chamber where coverslips modified to allow a continuous flow-through at a rate of 2 ml/min. Cuvettes were placed in a thermostatically controlled (37°C) holder in a Shimadzu RF5000 spectrofluorometer. BCECF fluorescence was measured by alternatingly exciting the dye at 500 and 440 nm at a constant emission of 530 nm (27). Fluorescence ratios were calibrated with 10 μM nigericin in 105 mM KCl (28), and pH was determined using a conversion program developed by G. Boyarsky (University of Texas, Galveston, TX). Cells were acid loaded by the application (10 min) and removal of 20 mM NH₄Cl (20 mM NaCl replaced with 20 mM NH₄Cl/NH₃) (29). Rates of recovery from this acid load (dpH/dt) were determined by evaluating the derivative of the slope of the pH trajectory of the intracellular pH, intervals of 0.05. In the presence of 10 μM HOE694 (provided by Dr. H. J. Lang, Hoechst AG), which selectively inhibits the NHE1 isoform at this concentration (30), there was no recovery from an acid load in HEK293, CCL, or COS cells, indicating that in a HEPES buffer, an extrusion from an acid load was primarily due to NHE1 activity. H⁺ efflux was determined as the product of the intracellular buffering capacity (HCO₃⁻/Na⁺) and Na⁺ dependence (31). The pH, recovery rate at the indicated pH values, represent the mean ± S.E. of the indicated number of separate cell transfections.

Oligonucleotide Synthesis, Poly(A)⁺ RNA Extraction, and RT-PCR—The design of oligonucleotide sequences was based on the available NHE1, NHE2, or NHE3 sequences obtained from GenBank, which are unique for different NHE isoforms but conserved between species for individual isoforms. The primer sequences for NHE1, based on NHE1 sequences from rat, and rabbit, were: 5′-ATAGGTTTCCATGATTTGAGAAGCATGTTGCGATTTGATGCTC-3′ (sense strand) and 5′-AGGCGCAGTGTGATTGATGCTGTC-3′ (antisense strand). The primer sequences for NHE2, based on NHE2 sequences from rat and rabbit, were: 5′-GAGAACATGTTGCGATTTGAGAAGCATGTTGCGATTTGATGCTC-3′ (sense strand) and 5′-AGGCGCAGTGTGATTGATGCTGTC-3′ (antisense strand). The primer sequences for NHE3, based on NHE3 sequences from rat and rabbit, were: 5′-GAGAACATGTTGCGATTTGAGAAGCATGTTGCGATTTGATGCTC-3′ (sense strand) and 5′-AGGCGCAGTGTGATTGATGCTGTC-3′ (antisense strand). POLY(A)⁺ RNA was isolated from cells by using FastTrack mRNA isolation kit (Invitrogen). RT-PCR was carried out with Poly(A)⁺ RNA (1 μg) isolated from the indicated cell types by using a Perkin-Elmer RNA-PCR kit.

RESULTS

Ga12 Inhibits NHE Activity in HEK293 and CCL39 Cells—Our previous study on G protein regulation of NHE activity examined the effect of mutationaly activated G protein α subunits expressed in HEK293 cells (19). These experiments identified several α subunits that coupled to the stimulation of NHE activity. They did not, however, reveal an α subunit inhibiting the NHE1 activity that could possibly mediate the GTP-dependent inhibition of NHE activity by somatostatin. NHE activity in HEK293 cells is very low even in the presence of serum, a condition which usually elevates NHE activity. We reasoned that perhaps this low steady-state exchange activity limited the potential inhibitory action of an α subunit. We therefore re-examined the effects of the mutationaly active α subunits that in our previous study did not regulate the steady-state exchange activity in HEK293 cells (19). This time, however, we determined their action on NHE activity stimulated by α13Q229L, one of the mutationaly active α subunits that increases NHE activity in HEK293 cells maintained continuously in serum (19). When the mutationaly active subunits α12Q227L, α10Q250L, and α12Q229L were coexpressed with α13Q229L, only α12Q229L reproducibly decreased two indices of NHE activity, the rate of recovery of pH following a transient acid load induced by NH₄Cl (Fig. 1, A and C) and the steady-state pH (Fig. 1B). In the absence of α13Q229L, expression of α12Q229L had no effect on NHE activity, α12Q227L, α10Q250L, and wild-type α12 did not have an effect on NHE activity (Fig. 1A) or pH (Fig. 1B) when expressed either in the absence or the presence of α13Q229L. Coexpression of α12Q229L with α13Q229L also inhibited the rate of H⁺ efflux, as determined by measuring the rates of pH change over the entire recovery period multiplied by the intrinsic cellular buffering capacity (Fig. 1D). Intrinsic buffering capacities, from pH 6.6 to 7.2, did not differ in HEK293 cells transfected with pcDNA vector, α13Q229L, or α12Q229L and α12Q229L (p > 0.2; n = 3, data not shown).

The low serum-stimulated exchange activity in HEK293 cells may limit the inhibitory effect of α12Q229L in the absence of α13Q229L. We therefore studied the action of α12Q229L alone in CCL39 fibroblasts maintained continuously in serum. At pH 6.75, serum-stimulated NHE activity in CCL39 cells (22.27 ± 1.53 dpH/dt × 10⁻⁶ pH/s) is significantly greater than that in HEK293 cells (8.09 ± 0.97; p < 0.01; n = 5). α12Q229L had no effect on the rate of pH recovery from an acid load in quiescent CCL39 cells maintained in serum-free medium for 18 h (data not shown). However, expression of α12Q229L, but not wild type α12, inhibited serum-stimulated rates of pH recovery (Fig. 2A). Additionally, α12Q229L reduced the serum-stimulated steady-state pH in CCL39 cells from 7.13 ± 0.02 (mean ± S.E.) to 6.99 ± 0.01 (n = 7 independent transfections). In quiescent CCL39 cells, expression of α13Q229L stimulates NHE activity (Fig. 2B). As was the case in HEK293 cells, in CCL39 cells coexpression of α12Q229L with α13Q229L reduced the rate of α13Q229L-stimulated pH recovery (Fig. 2B). The steady-state pH in serum-deprived CCL39 cells was 7.00 ± 0.03 in vector controls, 7.12 ± 0.02 in cells expressing α13Q229L, and 6.98 ± 0.02 in cells coexpressing α13Q229L and α12Q229L (n = 6 separate transfections). Hence, activation of α12 inhibits NHE activity in serum-stimulated, but not quiescent CCL39 cells.

Ga12 Stimulates NHE Activity in COS-7 Cells—During the course of these studies, Dhanasekaran et al. (20) reported that expression of α12Q229L stimulated NHE activity in COS-7 cells, an effect opposite to what we observed in HEK293 and CCL39 cells. To examine this paradox, we also determined the effects of α12Q229L on NHE activity in COS-7 cells. Because we anticipated that α12Q229L might inhibit the exchanger, we studied cells maintained continuously in serum. In agreement with Dhanasekaran et al. (20), however, we found that serum-stimulated NHE activity was increased in COS-7 cells expressing α12Q229L, but not wild type α12 (Fig. 3A). Additionally, the steady-state pH of 7.11 ± 0.01 in vector controls increased to 7.21 ± 0.02 in cells expressing α12Q229L (n = 3 separate transfections). To determine whether these paradoxical effects of α12 on NHE activity observed in CCL39 versus COS-7 cells could be explained by α12Q229L-induced changes in the intrinsic buffering capacity (βi), we determined the pH dependence of βi in COS-7 cells transfected with pcDNA or α12Q229L. Data
from three separate transfections indicated that the $p_H$ values (6.5–7.2) in cells expressing $\alpha_{12Q229L}$ were not significantly different from those of vector controls (Fig. 3B; $p > 0.2$). The $\alpha_{12Q229L}$-induced increase in $H^+$ extrusion, therefore, was not due to changes in the ability of COS-7 cells to buffer protons.

**Ga12 Differentially Regulates NHE Isoforms**—An explanation of the apparent cell-specific effects of $\alpha_{12}$ on NHE activity is that COS-7 cells might express an NHE isoform different from that in CCL39 fibroblasts. Five NHE isoforms have been cloned that differ in their tissue distribution, with expression of NHE1 being ubiquitous and the others sharing a more restricted pattern in epithelial cells. The activity of these isoforms is also differentially regulated. Activation of $G_s$-mediated signaling pathways has no effect on NHE1 in most cell types (23, 32, 33), although it can stimulate NHE1 in rat osteoblastic UMR-106 cells (34). In contrast, it inhibits the epithelial isoforms (35). The recent finding that mutationally activated $\alpha_{QL}$ potently inhibits NHE activity in COS-7 cells (20) suggests that the epithelium-specific isoforms might be expressed in these cells.

To study the effect of $\alpha_{12Q229L}$ on the activity of NHE isoforms, we used AP1 cells stably expressing NHE1, NHE2, or NHE3. AP1 cells are an NHE-deficient clone derived from Chinese hamster ovary cells (36). The kinetics and regulation of NHE isoforms expressed in these cells was reported previously (11). Because $\alpha_{12Q229L}$ regulated serum-stimulated NHE activity in both CCL39 and COS-7 cells, we studied its effects in AP1 cells maintained continuously in serum. In AP1 cells expressing NHE1, transient expression of $\alpha_{12Q229L}$, but not wild type $\alpha_{12}$, decreased serum-stimulated rates of $p_H$ recovery (Fig. 4A) and reduced steady-state $p_H$ from 7.12 ± 0.01 to 7.01 ± 0.01 (n = 4 separate transfections). In contrast, expression of $\alpha_{12Q229L}$, but not wild type $\alpha_{12}$, increased exchange activity in AP1 cells expressing NHE2 (Fig. 4B) or NHE3 (Fig. 4C). The steady-state $p_H$, also increased in the presence of $\alpha_{12Q229L}$, from 7.14 ± 0.02 to 7.23 ± 0.02 in AP1-NHE2 cells (n = 4) and from 6.95 ± 0.02 to 7.05 ± 0.01 in AP1-NHE3 cells (n = 4). As shown previously (11), we found that the steady-state activity of NHE3 was considerably less than that of NHE1 and NHE2. Although Ga12 appeared to induce an opposite shift in the set point of the $H^+$ "modifier

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**Fig. 1.** Effects of wild type and mutationally active $\alpha$ subunits on NHE activity in HEK293 cells. HEK293 cells were transfected with the indicated $\alpha$ subunits, and after 48 h cells were loaded with the pH-sensitive dye BCECF. Fluorescence ratios were calibrated to determine rate of $p_H$ recovery from an acid load at $p_H$ 6.75 (A), resting $p_H$ (B), time course of $p_H$ recovery from an acid load (C), and $p_H$-dependent $H^+$ efflux in vector control and cells expressing the indicated $\alpha$ subunits (D). Data represent the means ± S.E. of five to six separate transfections.
site” in NHE1 versus NHE2 and NHE3, the precise nature of kinetic changes could not be evaluated, because recovery rates were not determined at more acidic ranges (pH$_i$, 6.5). Hence, Ga12Q229L differentially regulated the activity of specific NHE isoforms; it inhibited NHE1 activity and stimulated NHE2 and NHE3 activities. In contrast to the isoform-specific action of α12Q229L, expression of α13Q226L stimulated the activities of NHE1, NHE2, and NHE3 (Fig. 4D). α13Q226L also increased the resting pH$_i$ of vector controls, from 7.12 ± 0.02 to 7.28 ± 0.03 in AP1-NHE1 cells, from 7.12 ± 0.01 to 7.23 ± 0.02 in AP1-NHE2 cells, and from 6.95 ± 0.05 to 7.06 ± 0.02 in AP1-NHE3 cells. These experiments showed that members of the Ga12/13 family have different effects on NHE isoforms. Ga13 couples to the stimulation of NHE1, NHE2, and NHE3, whereas Ga12 couples to the inhibition of NHE1 and to the stimulation of NHE2 and NHE3.

We next sought to determine whether α12Q229L stimulation of NHE activity in COS-7 cells could be due to the expression of NHE2 or NHE3 isoforms. RT-PCR was used to determine the expression of NHE isoforms in CCL39 and COS-7 cells with specific primers targeting NHE1, NHE2, and NHE3. Poly(A)$^+_f$ RNA isolated from AP1 cells stably expressing NHE1, NHE2, or NHE3 isoforms was used as a positive control. The amplified cDNA product of the predicted size of 320 base pairs for NHE1 was detected with RNA from all the cells examined: AP1-NHE1, CCL39, COS-7, and untransfected AP1 cells (Fig. 5A). Although wild type AP1 cells do not demonstrate NHE activity and protein (37), they do express NHE1 message. The AP1 clone was obtained by functional selection (36), and the functional deficiency in Na$^+$/H$^+$ exchange is very likely caused by point mutations that disrupt translation. Expression of NHE2 and NHE3 was not detected in CCL39 and COS-7 cells. The RT-PCR products of the predicted size of 450 base pairs for NHE2 and 710 base pairs for NHE3 were only observed with RNA from the positive control cellsof AP1-NHE2(Fig. 5B) and AP1-NHE3 (Fig. 5C). To further confirm that COS-7 cells do not express NHE2 and NHE3, we studied the effects of HOE694 on pH$_i$ recoveries from an acid load. Although HOE694 inhibits the activities of NHE1, NHE2, and NHE3, its affinity is much greater for NHE1 (K$_i$ of 160 nM) than NHE2 (K$_i$ of 5 μM) and NHE3 (K$_i$ of 650 μM) (30). COS-7 cells maintained continuously in serum were incubated with HOE694 (0.001–10 μM) for 10 min prior to acid loading (Fig. 5D). The K$_i$ of HOE694 inhibition was about 130 nM, and at 10 μM HOE694 pH$_i$ recovery in COS-7 cells was completely inhibited (Fig. 5D), suggesting the presence of only the NHE1 isoforn in COS-7 cells.

**DISCUSSION**

NHE activity is inhibited by the neurotransmitters somatostatin (23, 24) and dopamine (acting at a D$_2$ receptor subtype)
Because several GTPases have been shown to activate the exchanger (13–15, 19–22), our objective in the current study was to identify a G protein α subunit that might mediate receptor inhibition of NHE activity. We found that mutational activation of Ga12, a pertussis toxin-insensitive G protein, could inhibit stimulated Na⁺-H⁺ exchange activity in HEK293 and CCL39 cells. Thus, to date Ga12 is the only G protein found to play an inhibitory role in regulating NHE activity.

In contrast to its effect in HEK293 and CCL39 cells, Ga12Q229L stimulated NHE activity in COS-7 cells, a finding similar to that reported by Dhanasekaran et al. (20). One possible explanation for this opposite action of Ga12Q229L in CCL39 and CCL39 cells. Thus, to date Ga12 is the only G protein found to play an inhibitory role in regulating NHE activity.

In contrast to its effect in HEK293 and CCL39 cells, Ga12Q229L stimulated NHE activity in COS-7 cells, a finding similar to that reported by Dhanasekaran et al. (20). One possible explanation for this opposite action of Ga12Q229L in CCL39 and COS-7 cells is that these cells express different NHE isoforms, on which Ga12 may have different effects. To test this possibility, we studied the effect of mutationally activated Ga12 on specific NHE isoforms stably expressed in NHE-deficient AP1 cells. We found that Ga12Q229L did differentially regulate the NHE isoforms: it inhibited NHE1 activity but stimulated NHE2 and NHE3 activities. However, RT-PCR and HOE694 inhibition of pHᵢ recoveries from an acid load suggest that NHE1, but not NHE2 or NHE3, is expressed in COS-7 cells. Different signaling mechanisms, therefore, may contribute to the differential regulation of NHE1 by Ga12Q229L in CCL39 and COS-7 cells. Differences in GTPase-mediated signaling networks in fibroblasts and COS-7 cells have been reported previously (38). Additionally, although Gαs does not regulate NHE1 activity in fibroblast-derived cell lines (27, 32, 33), it couples to the inhibition of Na⁺-H⁺ exchange in COS-7 cells (20). Hence, we speculate that Ga12Q229L regulation of NHE1 activity is cell type-specific (fibroblasts versus COS-7 cells).

Our findings indicate that mutationally activated Ga12 and Ga13 have different effects on NHE1 activity: Ga13 stimulates NHE1 whereas Ga12 inhibits activated NHE1. Ga12 and Ga13 belong to a distinct pertussis toxin-insensitive G protein family (39). They share high sequence homology, and both are ubiquitously expressed (39, 40). In contrast to their differing actions on NHE1 activity, Ga12 and Ga13 have parallel effects on other cell processes. They both stimulate cell proliferation and induce neoplastic transformation (41–44). Both also stimulate ERK (extracellular signal-regulated kinase) (43) and JNK (Jun kinase)/SAPK (stress-activated protein kinase) (C-terminal Jun kinase) (45) activities. Additionally, both Ga12 and Ga13 couple to the stimulation of RhoA-mediated stress fiber forma-
Ga12 Regulates Na\(^+\)-H\(^+\) Exchange

22609

Vcd Ga12 differentially regulates the activity of NHE isoforms: it inhibits NHE1 activity, but stimulates NHE2 and NHE3 activities. Our studies with Ga12Q229L in fibroblasts and COS-7 cells further suggest that Ga12 regulation of NHE1 is probably cell type-specific. Ga12Q229L inhibits Na\(^+\)-H\(^+\) exchange in fibroblasts but stimulates Na\(^+\)-H\(^+\) exchange in COS-7 cells. We reported previously the stimulation of NHE1 activity by Ga13, the other member of the same G protein family. Thus, our findings on their function in NHE1 regulation demonstrate the only identified difference between Ga12 and Ga13 actions. In contrast to previous findings that cell transformation is associated with an increase in NHE1 activity, Ga12 induces transformation but inhibits NHE1 activity, suggesting that increases in NHE1 activity may not be essential for cell transformation.

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