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Gα13 Stimulates Na-H Exchange*

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Activity of the ubiquitous Na-H exchanger (NHE1) is regulated by a number of receptors with tyrosine kinase activity as well as by several classes of receptors coupled to heterotrimeric GTP-binding proteins. We previously demonstrated that the β_2-adrenergic receptor and other receptors that stimulate adenylyl cyclase by activating G_s stimulate NHE1 by a guanine nucleotide-dependent mechanism that is independent of receptor coupling to G_s. Now we report that a recently identified Gα subunit, α13, activates the exchanger. Transient expression of mutationally activated α13 constitutively stimulates Na-H exchange; moreover, an α13/α_z chimera, designed to respond to stimulation by G_i-coupled receptors, mediates stimulation of Na-H exchange by one such receptor, the dopamine$_2$ receptor. Mutationally activated α13, however, does not stimulate adenylyl cyclase activity or phosphoinositide hydrolysis, indicating that its action on NHE1 occurs independently of these two effector pathways. These findings reveal the first known signaling function of α13 and identify a new G protein involved in the regulation of NHE1.

Na-H exchangers comprise a family of electroneutral countertransport proteins participating in intracellular pH (pH_i)[1] homeostasis, cell volume regulation, and the transepithelial transport of Na^+ and acid-base equivalents (for reviews see Refs. 1 and 2). The only ubiquitously expressed Na-H exchange subtype, NHE1, functions primarily in controlling pH_i. Although a number of hormone and neurotransmitter receptors coupled to G proteins regulate NHE1 activity, the specific G proteins involved in this regulation remain to be identified. Several receptors that stimulate adenylyl cyclase, including the β_2-AR, parathyroid hormone, and prostaglandin E_1 receptors, stimulate NHE1 activity independently of a cholera toxin-sensitive G_s (3–5). We previously demonstrated that mutant β_2-ARs that are uncoupled from G_s due to deletions in amino acid residues within the third cytoplasmic domain (6) can activate the exchanger through a guanine nucleotide-dependent mechanism (7), suggesting that a previously unidentified G protein, distinct from G_s, may mediate this effect.

If a putative G protein couples the β_2-AR to NHE1, then one of the four Gα subfamilies (reviewed in Refs. 8–10) might contain a protein that regulates the exchanger. Of these, one subfamily seemed to contain the most likely candidates, two ubiquitously expressed Gα subunits of unknown function called α12 and α13 (11). Specific considerations ruled out Gα subunits in the other three families. Proteins in the α_s subfamily stimulate cAMP synthesis and are activated by cholera toxin, but neither cAMP nor cholera toxin stimulates NHE1 activity (3, 4, 12, 13). Although proteins in the α_i subfamily mediate activation of the exchanger by receptors for thrombin (14, 15) and fMet-Leu-Phe (16, 17), pertussis toxin blocks this activation by uncoupling receptors from α_i proteins but does not block β_2-AR stimulation of NHE1 (3, 4). α12 and α13 lack sites for modification by pertussis toxin (11). While proteins in the α_q subfamily stimulate phosphatidylinositol-specific phospholipase C (PI-PLC), they are not activated by the β_2-AR; moreover, protein kinase C, a downstream effector of PI-PLC, does not mediate β-adrenergic stimulation of NHE1.[2] We have now used a mutational analysis of several structural domains shared by GTPases to demonstrate that α13 stimulates both a rapid and prolonged activation of NHE1 activity.

MATERIALS AND METHODS

DNA Constructs and Transient Transfection—Mutationally active α_s (α_s-Q227L) and α_q (α_q-R183C) were constructed as previously described (18–20). Mutationally active subunits were constructed as described below and subcloned into the eukaryotic expression vector pcDNA-I (Invitrogen). α12-wt and α13-wt were gifts from Melvin I. Simon of the California Institute of Technology (11). α12 and α13 were excised from pMOB and subcloned into pcDNA-I into *Eco*RI and *Eco*RI and *Nsi*I sites. The cDNAs were then mutagenized as described (21) using the mutagenic primers GGC CTG AGA TCT CAG CGC CAG AAG TGG TTC CAG and GCG CTG AGA TCT CAG GCC GCC CAC ATC CAC CAT CTT for the α12 subunit and GGC CTG AGA TCT GAA CGG AAA CGC TGG TTT GAA and CCG TTC AGA TCT CAG GCC ACC TAC ATC AAC CAT TTT for the α13 subunit. The resulting mutant cDNAs (α12-Q229L and α13-Q226L) have new silent *Bgl*II restriction enzyme sites. Mutations were confirmed by DNA sequencing. A chimera of α_{13} and α_z cDNAs was produced using PCR by including the entire added sequence in the 3′-PCR primer, as described (21). The PCR product was then digested with *Eco*RV and *Nsi*I, and the resulting fragment was subcloned back into α13. The subcloned fragment was then sequenced. Wild type and mutant constructs were transiently transfected in human embryonic kidney (HEK)293 cells using DEAE-dextran (1 μg of cDNA/10^6 cells (20)).

Intracellular pH Measurements—Twenty-four h after transfection, cells were reseeded onto glass coverslips and maintained at 5% CO_2 in Earle's minimum essential medium supplemented with 10% fetal bovine serum. After an additional 24–48 h, cells were transferred to a nominally HCO_3^--free HEPES-buffered medium (3) and loaded with 1 μM acetoxymethyl ester derivative of the pH-sensitive dye 2,7-biscarboxyethyl-5(6)-carboxyfluorescein (BCECF, Molecular Probes) for 15 min at 37 °C. BCECF fluorescence was measured using a Shimadzu RF5000 spectrofluorometer by alternately exciting the dye at 500 and 440 nm at a constant emission of 530 nm. Fluorescence ratios were calibrated with 10 μM nigericin in 105 mM KCl (22). Cells were acid-loaded by the application (10 min) and removal of 20 mM NH_4Cl (23). Rates of recovery from this acid load (dpH_i/dt) were determined by

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[1] The abbreviations used are: pH_i, intracellular pH; NHE1, ubiquitous Na-H exchanger; β_2-AR, β_2-adrenergic receptor; HEK, human embryonic kidney; BCECF, 2,7-biscarboxyethyl-5(6)-carboxyfluorescein; D_2R, D_2-dopamine receptor; PI-PLC, phosphatidylinositol-specific phospholipase C; PCR, polymerase chain reaction; EIPA, ethylisopropylamiloride.

[2] M. B. Ganz and D. L. Barber, unpublished results.

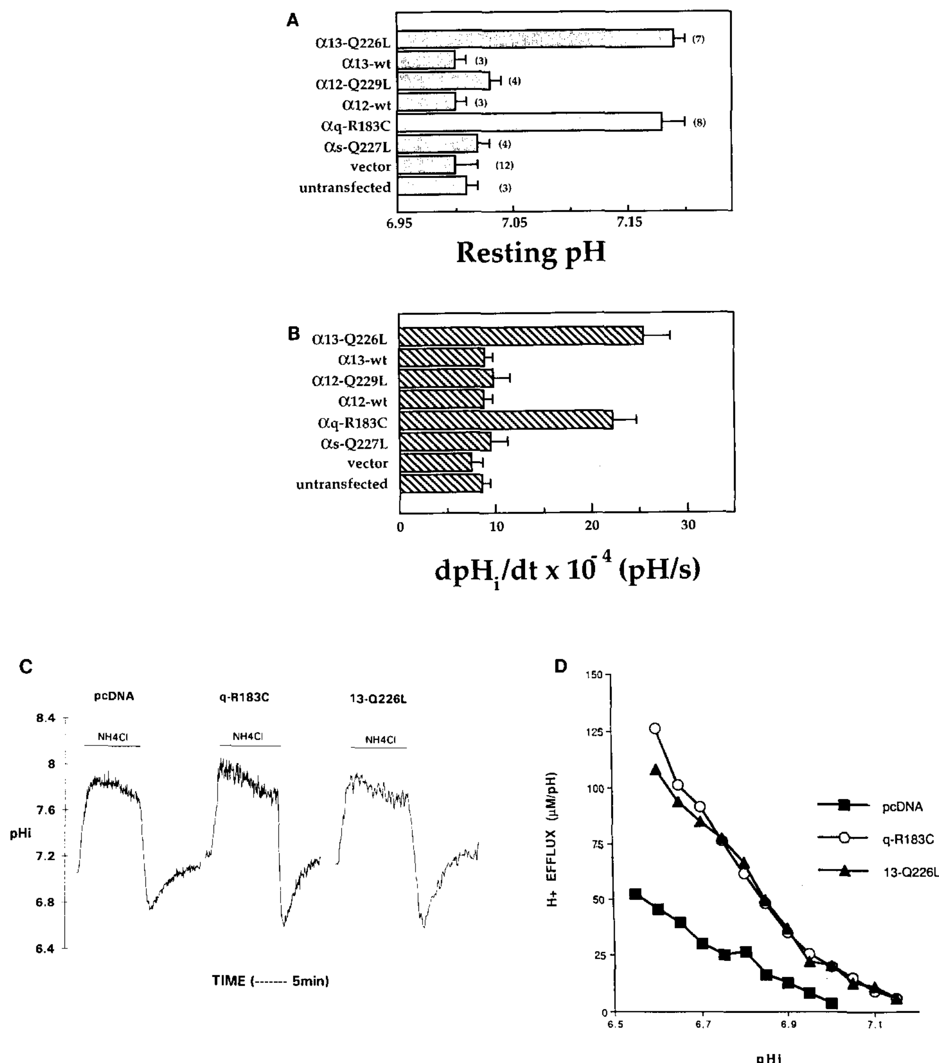


FIG. 1. **Effect of wild type and mutationally active α subunits on intracellular pH (pH_i) and acid extrusion.** HEK293 cells were transiently transfected with constructs of α subunits, and after 48–72 h cells were loaded with the pH-sensitive dye BCECF. Fluorescent ratios were calibrated to determine: *A*, resting pH_i; *B*, rate of pH_i recovery at pH_i 6.75 from an acid load induced by an NH_4Cl prepulse; *C*, time course of pH_i recovery from an acid load; and *D*, pH_i dependence of H^+ efflux in cells expressing vector alone (*pcDNA*) or expressing α_q-R183C or α13-Q226L. Data represent the mean ± S.E. of the indicated number of cell passages (untransfected) or the indicated number of transfections in duplicate and triplicate determinations.

evaluating the derivative of the slope of the pH_i tracing at pH_i intervals of 0.05. H^+ efflux was determined as the product of the intrinsic intracellular buffering capacity (HCO_3^-- and Na^+-dependent transport processes blocked (24)) and the pH_i recovery rate at the indicated pH_i values. Data represent the mean ± S.E. of the indicated number of cell passages (untransfected) or the indicated number of transfections in duplicate and triplicate determinations.

cAMP Accumulation—Twenty-four h after transfection cells were reseeded in 24-well plates and labeled with [^{3}H]adenine (2 μCi/ml) for 24 h. For measuring intracellular cAMP, cells were washed once with HEPES-buffered Dulbecco's modified Eagle's medium and incubated (37 °C for 30 min) in the same medium containing 1 mM 1-methyl-3-isobutylxanthine. Reactions were terminated by aspiration and the immediate addition of 5% ice-cold trichloroacetic acid (1 ml/well). Acid-soluble nucleotides were separated on ion-exchange columns as described (25), and results are expressed as (cAMP/cAMP + ATP) (× 10^3).

Phosphoinositide Hydrolysis—Twenty-four h after transfection, cells were reseeded onto 24-well plates and labeled with [^{3}H]myoinositol (2 μCi/ml) for 24 h. They were then washed once with HEPES-buffered Dulbecco's modified Eagle's medium and incubated at 37 °C for 1 h in the same medium containing 5 mM LiCl. Total inositol phosphate accumulation was assayed using Dowex columns as described (20), and results are expressed as counts per min of [^{3}H]inositol phosphate (10^3) divided by the sum of the counts per min in both the [^{3}H]inositol phosphate and [^{3}H]inositol fractions.

RESULTS AND DISCUSSION

If a specific Gα subunit mediates agonist stimulation of an effector pathway, a mutationally activated version of the same Gα should stimulate the same pathway in an agonist-independent fashion. Mutational substitution of amino acids at either of two conserved codons in α_s (18, 19), α_i (25, 26), and α_q (20, 27) inhibits the intrinsic GTPase activity of these proteins, thereby inducing their constitutive activation; expression of the mutant proteins leads to constitutive stimulation of the appropriate effector pathways (18–20, 25–27). We therefore expressed mutant Gα subunits in HEK 293 cells and assessed activity of the Na-H exchanger (Fig. 1). Expression of either α_q-R183C or α13-Q226L reproducibly increased two indices of Na-H exchange activity, the steady-state pH_i (Fig. 1*A*) and the rate of recovery of pH_i following a transient acid load induced by NH_4Cl (Fig. 1*B*). These changes were apparently caused by increased Na-H exchange, because pH_i recoveries were abolished in the absence of extracellular Na^+ or in the presence of an NHE1 blocker, ethylisopropylamiloride (EIPA, 50 μM) (results not shown). Neither resting pH_i nor the rate of pH_i recovery after an acid load was affected by mutationally active α_s (α_s-Q227L), wild type or mutant (Q229L) α12, or wild type α13 (Fig. 1, *A* and *B*).

Because long term expression of a mutant cDNA could affect pH_i by changing the ability of cells to buffer protons, we also assessed changes in the rate of H^+ efflux, as determined by measuring rates of pH_i change over the entire recovery range (Fig. 1*C*), multiplied by the intrinsic cellular buffering capacity. Intrinsic buffering capacities from pH_i 6.55 to 7.10 did not differ in untransfected cells, cells transfected with vector DNA alone, or cells expressing mutationally active α subunits (p > 0.2; n = 4). Expression of mutant α_q and α13 did, however,

increase the slope of H^+ efflux, from −12.0 ± 2.0 in vector-transfected controls to −23.2 ± 4.6 or −20.8 ± 2.3 $mM \cdot s^{-1}$ per pH unit in cells expressing α_q-R183C or α13-Q226L, respectively (Fig. 1*D*). When EIPA was applied at steady state to cells expressing vector, a small decrease in pH_i was observed (0.1–0.12 pH units). In contrast, application of EIPA at steady state to cells expressing α_q-R183C or α13-Q226 produced a larger relative decline in pH_i (0.3–0.33 pH units), suggesting that the steady-state H^+ efflux for the experimental group is actually an underestimate of the Na-H exchange activity.

The results in Fig. 1 suggest that prolonged activity of either α13 or α_q, but not of α_s or α12, stimulates Na-H exchange. The specificity of α13 action could be evaluated by determining effects of transiently transfected mutant α subunits on cAMP synthesis and PI-PLC in the same cells (Fig. 2). The pH_i response to activated α_q was expected, because this activated subunit stimulates PI-PLC activity (Fig. 2*A*) (20, 27, 28) and NHE1 activity is known to be elevated both by agonists acting on G_q-coupled receptors (29, 30) and also by phorbol esters (15, 31), which stimulate protein kinase C (32). Mutationally activated α13 stimulated neither detectable cAMP synthesis (Fig. 2*B*) nor detectable PI-PLC activity (Fig. 2*A*), suggesting that α13 regulates pH_i and the Na-H exchanger independently of either pathway. The data did not reveal a signaling function for α12; just as with pH_i and Na-H exchange (Fig. 1), neither wild type nor mutant (Q229L) α12 stimulated synthesis of either cAMP or inositol phosphates (Fig. 2). Constitutively active α13 could have stimulated Na-H exchange metabolically, for example by increasing production of H^+ via activation of the glycolytic pathway. The increased resting pH_i in cells expressing α13-Q226L, however, suggested metabolic acid production was not increased. Additionally, lactate production (33) in cells expressing activated α13 (43.7 ± 2.6 mmol/10^5 cells) was not significantly different from cells expressing vector alone (45.5 ± 4.7; $p > 0.2$; $n = 4$).

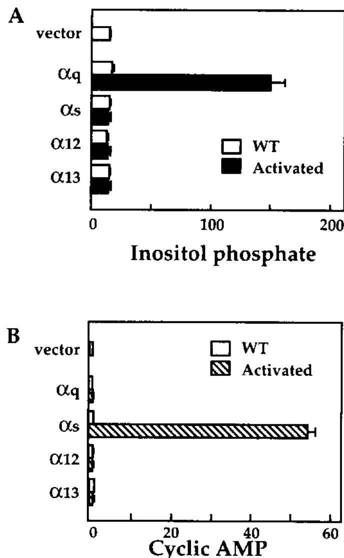
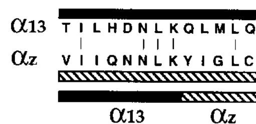
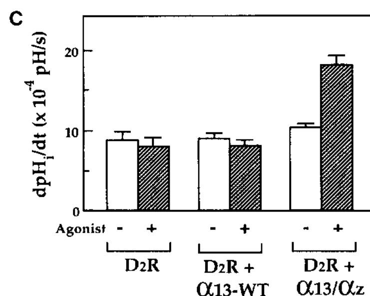


FIG. 2. **Effect of wild-type and mutationally active α subunits on phosphoinositide synthesis and cAMP accumulation.** HEK293 cells were transiently transfected with α subunit constructs and after 24 h were transferred to multiwell plates for the indicated assays. *A*, inositol phosphate accumulation in cells transiently expressing wild-type (*WT*, *open bars*) or constitutively activated α-subunits (*closed bars*). *B*, cAMP accumulation in cells transiently expressing wild-type (*open bars*) or constitutively activated α-subunits (*closed bars*). The data represent the mean ± S.E. of triplicate determinations in a representative experiment; three additional experiments gave similar results.

The effect of α13-Q226L on Na-H exchange could have reflected an indirect response, because the constitutively active Gα subunit was expressed for 48 h or more before activity of the Na-H exchanger was measured. To determine whether α13 can rapidly activate Na-H exchange, we constructed a chimeric $\alpha13/\alpha_z$ protein (Fig. 3*A*) designed to allow direct stimulation of the effector function of α13 by treating cells with a hormonal agonist. Based on evidence that receptor specificity of Gα subunits resides in their carboxyl termini (reviewed in Refs. 21 and 34), we previously reported (21) precedents for the $\alpha13/\alpha_z$ chimera. Substitutions of carboxyl-terminal residues from α_i, α_o, or α_z conferred on α_q the ability to respond to stimulation by the D_2R, a receptor that normally couples to G_i rather than to G_q. Accordingly, we surmised that substitution of 5 carboxyl-terminal residues from α_z for the corresponding 5 residues of α13 (Fig. 3) might produce a chimera that could respond to agonist activation of the D_2R by elevating activity of the Na-H exchanger. A stimulatory response mediated by the D_2R would be especially convincing, in view of our previous finding (4) that the D_2R in pituitary lactotrophs mediates inhibition of NHE1.

Acute activation of the $\alpha13/\alpha_z$ chimera by quinpirole, a D_2R agonist, stimulated the Na-H exchanger (Fig. 3). In cells co-expressing the recombinant D_2R and the $\alpha13/\alpha_z$ chimera, quinpirole rapidly increased pH_i (Fig. 3*B*) and doubled the rate of pH_i recovery from an acid load (Fig. 3*C*); this effect was blocked by the D_2R antagonist butaclamol (not shown). Quinpirole failed to increase pH_i or the rate of pH_i recovery in cells expressing either the D_2R alone or the D_2R plus wild type α13,





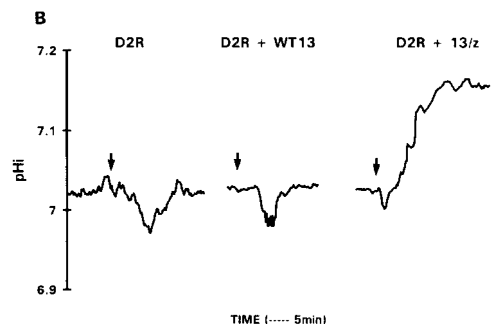


FIG. 3. **Structure and function of $\alpha13/\alpha_z$ chimera.** HEK293 cells were transfected with the D_2R or co-transfected with the receptor plus either wild type α13 or $\alpha13/\alpha_z$. After 48–72 h, pH_i determinations were made as described under "Materials and Methods." *A*, amino acid sequences of α13, α_z, and the $\alpha13/\alpha_z$ chimera. *B*, the D_2-agonist quinpirole (100 nM), added at indicated *arrows*, induced an increase in steady-state pH_i in HEK293 cells co-expressing the D_2R and $\alpha13/\alpha_z$ but induced a transient acidification in cells expressing the receptor alone or the receptor with wild type α13. *C*, effects of D_2R stimulation in the absence and presence of either wild type (*WT*) α13 or the $\alpha13/\alpha_z$ chimera on control rate of pH_i recovery from an acid load (*open bars*) and the recovery rate in the presence of quinpirole (*closed bars*). Data represent the mean ± S.E. of 4–7 transfections.

which presumably cannot couple to the D_2R; instead, in both cases quinpirole induced a small but significant transient decrease in pH_i (Fig. 3*B*). In other cells D_2R stimulation alone, in the absence of $\alpha13/\alpha_z$, inhibits Na-H exchange (4). The minimal effect produced by the D_2R in HEK293 cells not expressing $\alpha13/\alpha_z$ probably reflects the low steady-state activity of the exchanger in these cells, as suggested by the resting H^+ efflux in Fig. 1*D*.

In summary, we have shown that α13 (activated either by a GTPase-inhibiting mutation or through an altered carboxyl-terminal amino acid sequence that allows it to be stimulated by the D_2R) can stimulate activity of the Na-H exchanger. Our results do not establish whether or not α13 exerts a direct effect on the exchanger. Epidermal growth factor and α-thrombin stimulate NHE1 by phosphorylating a common set of serine residues located at the COOH terminus of the exchanger (35). Receptor-stimulated exchange activity may therefore involve a signaling cascade that includes a serine kinase upstream of the exchanger. Our findings do suggest, however, the possibility that agonists for the β_2-AR and other G_s-coupled receptors that activate NHE1 (3–5, 7) may couple to G13 as well as to G_s. Moreover, the acute and prolonged effects of α13 could indirectly influence a variety of important cell functions in which Na-H exchange plays an integral role. These include growth (36), differentiation (37, 38), and secretion (39, 40). We are now testing these interesting possibilities.

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