Communication

Ras-dependent Signaling by the GTPase-deficient Mutant of Ga

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Ga
and Ga
regulate diverse responses through the small GTPases Ras, CDC42, Rac, and Rho. Whereas they activate similar responses in many different cell types, they also activate more specific and critical signaling pathways in other cell types. In COS cells, in which both Ga
and Ga
stimulate Na+/H+ exchange, they do so by activating different signaling pathways. Here we report that the differential recruitment of specific small GTPases by Ga
and Ga
defines the molecular basis for their functional differences. We have observed that the stimulation of Na+/H+ exchange by the GTPase-deficient mutant of Ga
(Ga
QL) requires a functional Ras and is independent of Rac/CDC42 and Jun kinase signaling module. By contrast, the stimulation of Na+/H+ exchange by Ga
QL requires a functional Rac/CDC42 and the Jun kinase signaling module. Our results also indicate that Ga
QL-Ras stimulation of Na+/H+ exchange involves a D09-sensitive phospholipase and protein kinase C. These studies, for the first time, describe a novel Ga
-specific signaling pathway involving Ras, phosphatidylinositol hydrolysis, and protein kinase C in the regulation of Na+/H+ exchange.

G proteins\(^1\) mediate the signal transduction between heptahelical receptors and intracellular effectors. Of the signaling responses regulated by the four subfamilies of G proteins, the responses regulated by the Ga family of G proteins are beginning to be understood only recently (1). In a previous report, we have shown that the GTPase-deficient activated mutants of Ga
(Ga
QL) and Ga
(Ga
QL) differentially stimulate the Na+/H+ exchange in COS-1 cells (2). The stimulation of Na+/H+ exchange by Ga
is protein kinase C (PKC)-dependent whereas the stimulation by Ga
is PKC-independent. It has also been shown that whereas Na+/H+ exchange is stimulated by Ga
in COS cells, it is inhibited in HEK293 and CCL39 cells (3). Studies from several laboratories including ours have established that these G proteins are also involved in regulating several other responses (1). Whereas Ga
and Ga
are equally effective in activating most of these signaling responses they differ in their effect on Na+/H+ exchange. Thus, it appears that whereas Ga
and Ga
activate a similar set of responses, in different cell types or at different physiological contexts in the same cell type, they may regulate distinct sets of responses. Strong support for this argument comes from the recent Ga
gene knock-out studies carried out by Offermanns et al. (4), which suggest that Ga
and Ga
regulate distinct, non-complementary signaling pathways during mammalian embryogenesis. Defining the molecular basis for the differential signaling by Ga
and Ga
will prove critical to the understanding of the unique and critical responses regulated by these two G proteins, respectively. Therefore, we examined whether the differential regulation of Na+/H+ exchange by Ga
and Ga
can be used to define the molecular basis for the functional differences between them.

Na+/H+ exchange is mediated by a distinct family of transmembrane proteins known as Na+/H+ exchangers (NHEs). Both phosphorylation-dependent and -independent mechanisms are involved in the stimulation of NHEs (5, 6). In addition, the a-subunits of heterotrimeric G proteins as well as small GTPases have been shown to regulate the activities of NHEs (2, 3, 7–10). Based on the observations that (i) Ga
and Ga
stimulate both NHEs and Jun N-terminal kinases/stress-activated protein kinases (JNK), (ii) JNKs are activated by osmotic stress, (iii) JNK activation is involved in cell volume regulation, and (iv) NHEs are stimulated during hyperosmotic stress-induced regulatory volume increase, we proposed that the activation of NHE and JNK might be an integrated response regulated by Ga
(1). Although the studies by Hooley et al. (9) demonstrating that Ga
stimulation of Na+/H+ exchange is CDC42-MEKK-dependent in CCL39 fibroblasts confirm to this hypothesis, the molecular basis for PKC-sensitive stimulation of Na+/H+ exchange by Ga
has remained unclear. We present here our studies indicating that the signaling pathway leading to the stimulation of Na+/H+ exchange by Ga
has remained unclar. The dominant negative constructs, kinase-deficient pEBG KR-JNK (K116R) and pIFSET KR-JNK (K52R), were gifts from Dr. G. L. Johnson. The expression vector pcDNA3.1(-) KR-JNK1 was constructed by ligating the BamH1-HindIII fragment from pIFSETB-KR-JNK1 into the BamH1-HindIII site of pcDNA3.1(-). The dominant negative phenotypes of these constructs have been previously described (13, 14). The procedures for the transfection of COS cells and kinase assays have been previously described (2, 11). Cellular growth was determined with the fluorescent pH indicator BCECF-AM (Molecular Probes, Eugene, OR) according to the previously published procedures (2). Acid-
results and discussion

Our previous studies have shown that Go_{12} stimulates JNK and Na\(^+\)/H\(^+\) exchange activities in COS-1 cells. In these cells, the activation of Na\(^+\)/H\(^+\) exchange by Go_{12} is dependent on PKC whereas the activation of JNK is dependent on the presence of the small GTPase, Ras (2, 11). It has also been shown that Go_{12} activation of JNK involves other small GTPases such as Rac, CDC42, and the Ser/Thr kinase MEKK (15, 16). Since JNK has been suggested to be a candidate kinase involved in the regulation of cell volume (17) and the regulatory increase in cell volume is in some cases critically regulated by Na\(^+\)/H\(^+\) exchange (18), we investigated whether the stimulation of Na\(^+\)/H\(^+\) exchange by Go_{12} is mediated by JNK. COS-1 cells were cotransfected with plasmid vectors containing the cDNA inserts encoding a kinase-deficient mutant of JNK1 (KR-JNK1) and Go_{12}QL. The transfectants were analyzed for Na\(^+\)/H\(^+\) exchange activity. Similar cotransfection studies were carried out with Go_{13}QL for comparison. As indicated in Fig. 1, the expression of constitutively activated, GTPase-deficient Go_{12} or Go_{13} stimulated the Na\(^+\)/H\(^+\) exchange as indicated by the recovery rate of cell pH from an acid load. The coexpression of KR-JNK had no effect on Go_{12}QL-stimulated Na\(^+\)/H\(^+\) exchange whereas it had a strong inhibitory effect on Go_{13}QL-stimulated Na\(^+\)/H\(^+\) exchange.

JNK1 is activated through phosphorylation by an upstream Ser/Thr kinase known as Jun N-terminal kinase kinase. When the dominant negative mutant of JNKK (KR-JNKK) was co-transfected along with Go_{12}QL or Go_{13}QL, only the activity stimulated by Go_{13}QL was inhibited whereas that stimulated by Go_{12}QL remained unaffected (Fig. 1). The sensitivity of Go_{13}QL to JNKK and JNK is not surprising in light of the recent observations by Hooley et al. (9), demonstrating that MEKK, an upstream kinase of JNKK-JNK, is involved in the stimulation of NHE1 by Go_{13}QL. However, the finding that Go_{13}QL stimulation of Na\(^+\)/H\(^+\) exchange is not mediated by the JNKK-JNK-signaling module is novel and significant since it strongly suggests that Go_{13}QL stimulates Na\(^+\)/H\(^+\) exchange through a distinct JNK-independent signaling pathway.

Activation of the MEKK-JNKK-JNK signaling module (JNK module) is primarily mediated by Rac and CDC42, members of the Rho family of GTPases (12, 19). However, it has been recently shown that Go_{12}QL stimulates JNK through Ras, Rac, or CDC42 depending upon the cell type in which it is expressed (11, 15, 16). Furthermore, it has been shown that the activated mutant of Go_{12} can interact with both Ras- and Rac-dependent signaling pathways in NIH3T3 cells in the activation of the cell-proliferative response (20). In addition, Go_{12}QL has been shown to interact with Rho (21). In this context, it is noteworthy that Go_{12}QL stimulation of Na\(^+\)/H\(^+\) exchange has been shown to be dependent upon CDC42 as well as Rho (9). Hence we sought to examine whether the JNK-independent stimulation of Na\(^+\)/H\(^+\) exchange by Go_{12}QL is through a distinct small GTPase(s) different from the one being recruited by Go_{13}QL. COS cells were cotransfected with the dominant negative, competitively inhibitory mutants of Ras, Rac, or CDC42 together with Go_{12}QL or Go_{13}QL. As shown in Fig. 2, the coexpression of the dominant negative Rac completely inhibited Na\(^+\)/H\(^+\) exchange stimulated by Go_{12}QL whereas the coexpression of dominant negative Ras or CDC42 did not. In contrast, the coexpression of dominant negative Rac or CDC42 inhibited the Go_{13}QL-stimulated Na\(^+\)/H\(^+\) exchange activity whereas the dominant negative Ras failed to have any effect. It is worth noting that the expression of the activated mutants of both Rac (RacV12) and CDC42 (CDC42V12) by themselves has been shown to stimulate Na\(^+\)/H\(^+\) exchange in a JNK module-dependent manner in CCL39 cells (9). Taken together, our results suggest that Go_{12}QL stimulates Na\(^+\)/H\(^+\) exchange through a Ras-dependent but JNK-independent mechanism whereas Go_{13}QL stimulates Na\(^+\)/H\(^+\) exchange through a Rac/CDC42 and JNK-dependent signaling pathway. In this context, it is significant to note that although Go_{12}QL and Go_{13}QL display differential sensitivity to KR-JNK/KR-JNKK in the regulation of Na\(^+\)/H\(^+\) response, they show equal sensitivity to these dominant negative kinases in the regulation of JNK activity (data not shown). Thus it appears that the signaling by Go_{12} bifurcates at the level of Ras, one leading to the stimulation of Na\(^+\)/H\(^+\) exchange and the other to the activation of JNK.

If the stimulation of Na\(^+\)/H\(^+\) exchange by Go_{12}QL is mediated by Ras, then Ras itself should be able to activate the Na\(^+\)/H\(^+\) exchange. Also, the exchange activated by Ras should be sensitive to PKC, since Go_{13}QL stimulation of Na\(^+\)/H\(^+\) exchange is PKC-dependent. Although the ability of Ras and its downstream effector kinases such as Raf and ERK has been shown to stimulate Na\(^+\)/H\(^+\) exchange (6, 8), the role of PKC in Ras-mediated Na\(^+\)/H\(^+\) exchange is not yet defined. Hence, we investigated the ability of Ras to stimulate Na\(^+\)/H\(^+\) exchange in the presence or absence of the endogenous PKC activity.
COS-1 cells were transfected with the activated mutant of Ras (Ras V12). In a duplicate set of transfectants, the endogenous PKC was depleted by the chronic treatment with PMA (500 ng/ml) for 24 h. Go_{12}QL and Go_{13}QL transfectants were also subjected to similar treatment procedures. The Na\(^+/H^+\) activities of the transfectants were determined. As indicated in Fig. 3, expression of RasV12 leads to an increased Na\(^+/H^+\) exchange activity of COS cells, similar to those of Go_{12}QL and Go_{13}QL. More significantly, the expression of RasV12 failed to stimulate Na\(^+/H^+\) exchange when PKC was depleted in these cells similar to Go_{12}QL (Fig. 3). Consistent with our previous observations, depletion of PKC activity did not have any inhibitory effect on the Go_{13}QL-stimulated Na\(^+/H^+\) exchange (2). Taken together, these results strongly point to Go_{12}QL stimulation of Na\(^+/H^+\) exchange being mediated by Ras through a PKC-sensitive signaling pathway. It is significant that the rate of Na\(^+/H^+\) exchange activated by Ras is higher than the rates with Go_{12} or Go_{13}. This may be indicative that Ras can stimulate Na\(^+/H^+\) exchange by more than one pathway including the ERK pathway. The observation that the Ras-stimulated Na\(^+/H^+\) exchange is only partially inhibited by the chronic PMA treatment is also consistent with this view.

Since Ras can activate the ERK signaling module and ERK has been shown to be involved in the stimulation of Na\(^+/H^+\) exchanger (5, 8), it can be argued that the observed reduction in Na\(^+/H^+\) exchange is through the modulation of ERK activity by PKC. To examine whether the PKC sensitivity is a function of an altered ERK activity, we determined both the Ras-stimulated ERK2 activities of the transfectants following PKC depletion. As shown in Fig. 4, A and B, the depletion of endogenous PKC did not inhibit either of the ERK2 activities. On the contrary, the depletion of PKC has resulted in a small increase in the activation of ERK2 (Fig. 4, A and B), which is consistent with the observation that the chronic PMA treatment of different cell lines leads to the depletion of a growth-inhibitory isoform of PKC (22). The most significant conclusion from these experiments is that the reduction of Na\(^+/H^+\) exchange observed in Ras and Go_{12} transfectants can only be attributed to the depletion of endogenous PKC. These results, together with the observation that Go_{12}QL expression does not stimulate ERK2 activity in COS cells (Fig. 4A (16)), suggest that Go_{12}QL-stimulated, Ras-mediated, and PKC-sensitive Na\(^+/H^+\) exchange does not involve ERK activation.

Several studies have indicated a role for PKC in Ras signaling although its implication in Ras-mediated Na\(^+/H^+\) exchange is a novel one. The presence of multiple isoforms of PKC that have different effects on Ras signaling has added immense complexity in resolving the relationship between PKC and Ras in any specific signaling pathway (23–25). The PKC sensitivity of the Go_{12}QL-Ras-mediated Na\(^+/H^+\) exchange response evidences that a diacylglycerol-sensitive PKC isoform is involved. Diacylglycerols (DAGs) are produced by the action of phosphatidylinositol-specific phospholipase C (PI-PLC) that hydrolyzes phosphatidylinositol to inositol phosphates and DAGs. Since Go_{12}QL expression in COS cells does not lead to any increase in inositol phosphate levels or in PI-PLC activity, it is unlikely that PI-PLC is involved in Go_{12}QL-Ras stimulation of Na\(^+/H^+\) exchange. DAGs can also be produced by alternate signaling pathways involving phospholipases such as phosphatidylcholine-specific phospholipase C (PC-PLC), phospholipase D (PLD), or phospholipase A2 (25). Interestingly, Ras has been known to modulate the activities of both PC-PLC (26) and PC-PLD (27). Therefore, we used tricyclohexylphosphine-9-yl xanthogenate, commonly known as D609, an inhibitor of PC-PLC and PC-PLD (28, 29), to discern whether Go_{12}QL-Ras coupling to PKC (in stimulating the Na\(^+/H^+\) exchange) involves any of these phospholipases.

As shown in Fig. 5, when the cells expressing Go_{12}QL or RasV12 were treated with D609 (10 μg/ml) for 1 h, the Na\(^+/H^+\) stimulation was completely inhibited. In a striking contrast, the Na\(^+/H^+\) activity stimulated by Go_{12}QL was unaffected, demonstrating that the signaling pathway involving Ras and PC-PLC is specific to Go_{12}QL. The observations that
the predominant form of PMA-sensitive PKC expressed in COS cells is PKCα and that PKCα is also responsive to DAGs derived from PC (30) suggest that the Go12-Ras-phosphatidylycholine phospholipase signaling nexus presumably involves PKCα in stimulating Na"^+"/H"^+" exchange in COS-1 cells. At present, we are unable to distinguish between PLC and PLD in this pathway because of the recently observed lack of specificity of D609 to either of these lipases (28, 29). Furthermore, the transient expression system as well as the dynamic interconversion of various lipid intermediates in this pathway precluded us from identifying the D609-sensitive phospholipase activated by Go12 through Ras. Nevertheless, the identification that a D609-sensitive phospholipase is involved in Go12 signaling is a significant one, and this property clearly distinguishes Go12 from Go13. At present, the mechanism by which Go12 selectively stimulates a specific Ras signaling pathway without having a stimulatory effect on an ERK pathway is not fully understood. Similarly, it is still unclear how the JNK activated by Go12QL is not coupled to the stimulation of Na"^+"/H"^+" exchange whereas the JNK activated by Go13QL is coupled. It is likely that response-specific hard wiring of the Go12-signalng pathway and/or the ability of Go12 to generate parallel signals that can block other signaling "noises" may play a major role in insulating the Go12-Ras-PC-PLC/PC-PLD pathway from being affected by the random drifts of Ras into other signaling pathways. Perhaps, the "parallel signals" emanating from Go13 may also suppress the JNK-dependent activation of Na"^+"/H"^+" exchange. Such a mechanism would be analogous to the observations reported by Barber and colleagues (3) in which the expression of Go13QL inhibited the Na"^+"/H"^+" exchange activated by Go12QL. In addition, the recent observation that Go12 and Go13 activate JNK while concomitantly causing an inhibition of mitogen-activated protein kinase/ERK kinase and ERK activities through independent mechanisms (16) points to the multiplex signaling capabilities of these α-subunits.

In this context, it should be pointed out that the Na"^+"/H"^+" exchange activities measured here do not define the activity of a single type of the NHEs. Hence, it is possible that these α-subunits may be involved in the regulation of different NHEs. At present, the need for the presence of two different mechanisms regulated by two closely related but distinct Go-subunits in a single cell type is not known. It is likely that the presence of different NHEs at different locations of the cell (e.g. apical versus basolateral surfaces) or the physiological need to activate specific NHEs at different physiological contexts (for e.g. regulatory volume increase versus regulatory volume decrease) may require the presence of independent signaling pathways.

It is significant to note here that Go13 stimulation is mediated by Ras through the PC-phospholipase/PKC network whereas Go12 stimulation of Na"^+"/H"^+" exchange is mediated by the Rac/CDC42-JNK signaling module. Based on these observations, it can be predicted that Go12 is primarily involved in Ras-dependent responses such as mitogenic pathways whereas Go13, which avidly interacts with Rac/CDC42, may be more involved in the regulation of Rac-dependent responses. In this context, it is worth noting that the fibroblasts derived from Go13−/− mouse embryos did not show any decrease in ligand-induced DNA synthesis or cell proliferation although both the alleles of wild-type Go12 were present (4). However, these cells were found to be less responsive to thrombin-stimulated chemo motions involving cell motility. Since CDC42 and Rac are critically involved in cytoskeletal responses such as filopodia and lamellipodia formation, it is likely that Go13 deficiency leads to a functional uncoupling between the cell surface receptors and Rac and CDC42. Although the mechanisms by which Go12 and Go13 transmit their signals to the respective low molecular weight GTPases are presently not known, it is likely that they interact with Rac- or Rac-specific guanine nucleotide exchange factors or guanine nucleotide dissociation inhibitors. Identifying the molecules that couple Go12 and Go13 to the Ras and Rho family of GTPases may finally define the functional and evolutionary significance of both the similar and divergent signaling pathways regulated by these two G proteins.

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FIG. 5. D609 inhibits Go13QL- and RasV12-stimulated Na"^+"/H"^+" exchange. COS-1 cells were transfected with Go13QL, Go12QL, or RasV12 along with the empty vector (pcDNA3). At 60 h following transfection, one set of cells in each group was treated with D609 (10 μg/ml) for 1 h. The rate of pH recovery from an acid load (dpH/min) of these transfectedants was determined. Shown are mean ± S.E. values from three independent experiments.