Atrial natriuretic peptide (ANP) inhibits the proliferation of many cells, in part through interfering with signal transduction enacted by G protein-coupled growth factor receptors. Signaling interactions between ANP and the G protein-coupled growth factor receptor ligand, endothelin-3 (ET-3), regulate astrocyte proliferation at a very proximal but undefined point. Here, we find that ANP inhibits the ability of ET-3 to activate Go_i and Go_q in these cells. ANP stimulated the translocation of endogenous regulators of G protein-signaling (RGS) proteins 3 and 4 from the cytosol to the cell membrane, and enhanced their association with Go_i and Go_q. ANP effects were significantly blocked by HS-142–1, an inhibitor of guanylate cyclase activation, or by ET-3, KT5823, an inhibitor of cyclic GMP-dependent protein kinase (PKG). This represents a novel cross-communication between PKG and RGS proteins in a PKG-dependent fashion, and the expressed PKG (in the absence of ANP) also stimulated RGS phosphorylation. A novel cross-talk between PKG and RGS proteins is stimulated by ANP and leads to the increased translocation and association of RGS proteins with Go_q. The rapid inactivation of G proteins provides a mechanism by which ANP inhibits downstream signaling to the cell proliferation program.

Growth factors generally initiate cell proliferation upon binding to their cell membrane receptors and enacting signal transduction to the nucleus (1). Both tyrosine kinase and G protein-coupled growth factor receptors (GPCR) utilize various signal transduction pathways to accomplish this (1, 2), but the best understood involves the stimulation of the extracellular signal-regulated kinase cascade (1, 3). An in vitro model that details GPCR signaling is astrocyte proliferation; here, the mitogen endothelin-3 (ET-3) binds a heptahelical ETB receptor, activates Go_q and Go_i, and triggers downstream signaling through a variety of effectors (4), ultimately leading to extra-cellular signal-regulated kinase activation (5). Extracellular signal-regulated kinase then increases the transcription of Egr-1, which in turn binds to the promoter and stimulates the transcription of the astrocyte growth factor basic fibroblast growth factor gene (6). This sequence leads to glial cell cycle progression (7) and astrocyte division. Equally important, the anti-mitogen, atrial natriuretic peptide (ANP) inhibits many steps, which are stimulated by ET-3. The interactions between these two peptides probably begin at an undefined but proximal level of signal transduction (8), perhaps at the level of G protein activation.

G protein activation occurs rapidly after ligand binding to GPCRs. Ligation results in the dissociation of GDP from the G protein heterotrimERIC complex, whereupon GTP binds to the Go subunit in its active state, and the Goβγ subunit complex dissociates; either Go or Goβγ subunits can stimulate downstream effectors (reviewed in Ref. 9). Go subunits have an intrinsic GTPase activity that hydrolyzes GTP to GDP. Go-GDP has a higher affinity for Goβγ and their association terminates Goβγ signaling (reviewed in Ref. 10).

The inhibition of G protein activity can arise from several known mechanisms. Increased activity of protein kinases A, C, or G protein receptor kinases results in the phosphorylation/desensitization of GPCR and down-regulation of G protein activity (11, 12). Increased GoGTPase activity can be stimulated by effectors, such as phosphodiesterase or phospholipase C (13, 14). Recently, regulators of G protein signaling (RGS) proteins have been isolated and shown to stimulate GTPase activity, i.e. behave as GTPase-activating proteins for Go subunits (15–19). There are 20 RGS proteins currently identified in eukaryotic cells (20), and they show some specificity of function; different RGS proteins preferentially bind to different Go subunits (20, 21). Much of our knowledge of the function of these proteins comes from reconstituted systems and the modulation and roles of endogenous RGS in primary, nontransfected cells is not as well understood. Here we report that ANP inhibits the ET activation of Go_q and Go_i in primary cultures of astrocytes. This action of ANP is potentially mediated in part through alterations of RGS protein localization and function and occurs via the activation of cyclic GMP-dependent protein kinase (PKG). This represents a novel cross-communication between signal transduction systems in these cells.

MATERIALS AND METHODS
Cell Preparation and Materials—Primary cultures of fetal rat diencephalic astrocytes were prepared and maintained as described previously (22). Cells were typically used in their second passage. Antibodies and substrate for kinase activity were from Santa Cruz Biotechnology.
ANP Inhibits G Protein Activation

Growth factors were from Calbiochem or Sigma. LipofectAMINE was from Life Technologies, Inc. RGS-specific antiserum was raised in a rabbit against recombinant RGS protein and recognizes a 75-kDa protein that co-migrates with recombinant and in vitro translated RGS3. The RGS4 antibody is also a polyclonal, rabbit antibody raised against the N terminus of RGS4 coupled to limpet hemocyanin. The antibody recognizes a 30-kDa protein that co-migrates with recombinant RGS4, and was kindly provided by Dr. Kirk Druey, NIH.

G Protein Activation Assay—Dishes (100 mm) of cultured astrocytes were incubated with 10 nM ET-3 in the presence or absence of 10 nM ANP or C-ANP4–23 for 2 min. Cell membranes from astrocytes were then prepared and analyzed for lack of cytosol (23), and G protein activation brought on by the original whole cell incubation conditions was determined as described previously (24). Membrane aliquots were resuspended and incubated with 30 nM [35S]GTPyS (NEN Life Science Products) for 5 min at 30 °C. Incubations were terminated, and cell lysates were added to 2 μl of nonimmune serum preincubated with a 10% suspension of pansorbin cells (Calbiochem). After centrifugation, the supernatant was incubated with Gq or Gi α subunit antibody (Calbiochem) and preincubated with protein A-Sepharose. Immunoprecipitants were boiled with SDS, and GTPyS binding was quantified by scintillation counting; each condition was prepared in duplicate, and the data were combined from three separate experiments.

G Protein Association Studies—Whole astrocytes were first incubated for 2 min at 37 °C with ANP, ET-3, or both peptides followed by membrane isolation from each experimental condition. Antibodies to RGS3 and -4 (diluted 1:50) were complexed to protein A-Sepharose beads, and 100 μl of membranes from discrete incubation conditions were added and incubated for 2 h at 4 °C. After microcentrifuging, pellets were solubilized with gel-loading buffer, boiled, and then separated on a 10% SDS-PAGE gel. The separated protein was transferred to a membrane and blotted with antibodies to Gq and Gi proteins, using the ECL Western blot kit (Amersham Pharmacia Biotech).

GTPase Assays—GTPase activity measurements in rat astrocyte cell membranes were carried out as described (26). First, whole astrocytes were lysed without (medium alone) or with ET-3, ANP, or both peptides, for 2 min, to affect RGS translocation, and then membranes from each condition were isolated. Some cells were also transfected to express the active catalytic subunit of the PKG I, and these membranes were also isolated in parallel with the other conditions. Equal amounts of cell membrane proteins from each incubation condition were then exposed to the same experimental treatment under which the membranes were isolated, for a time course of GTPase. The PKG membranes were incubated for 5 min with ET-3 + NP, and then the cells were washed and lysed, and the membranes were isolated. Increased binding of labeled GTP to the immunoprecipitated Gq (A) and Gi (B) subunits was seen in the cell membranes from cells treated with ET-3, determined as described under “Materials and Methods.” The data are mean ± S.E. from three experiments combined. *, p < 0.05 for control versus ET-3 by analysis of variance plus Scheffe’s test; +, p < 0.05 for ET-3 plus ANP or C-ANP 4–23.

G Protein Phosphorylation Studies—Cells were labeled with [35S]methionine and [35S]cysteine, some in the presence of KT5823 or ET-3, and RGS translocation was determined.

RESULTS

G Protein Activation by ET-3 Is Inhibited by ANP—Our previous studies led us to postulate that ET-ANP interactions occur proximally. We now find that in membranes from primary cultures of nonmanipulated astrocytes, ET-3 directly stimulates an increase in Gq and Gi activity, determined by enhanced binding of GTPyS to these subunits (Fig. 1). We also found that the antigrowth factor, ANP, significantly inhibits ET-3-stimulation of Gq or Gi activity by 51 and 56%, respectively. Furthermore, C-ANP 4–23, a specific ligand for the atrieric peptide clearance receptor (NPCR) (27), was 50–65% as potent as ANP in inhibiting Gq and Gi protein activation by ET-3. ANP binds both guanylate cyclase (GC) receptors and NPCR with equal avidity and hence cannot be used alone to determine which receptor mediates the actions of ANP. These results suggest that both the guanylate cyclase and NPCR receptors contribute to G protein inactivation. Because the NPCR ligand is not as potent as ANP in suppressing G
protein inactivation, the two receptor subtypes may utilize different mechanisms to accomplish this.

**ANP Stimulates RGS Translocation**—As a potential mechanism, we determined whether ANP could stimulate the translocation of RGS to the cell membrane. RGS proteins are believed to down-regulate G protein activation by increasing the GTPase activity of $G_{\alpha}$ and $G_{\alpha_q}$ (20), and for certain RGS proteins this may require the RGS protein to translocate to the cell membrane (28). Some RGS proteins may be constitutively associated with the membrane, whereas others are translocated upon the appropriate stimuli.

By immunoblot, the astrocytes produce RGS3 and RGS4 proteins (data not shown). We found that ANP significantly stimulated the translocation of both RGS3 and RGS4 to the cell membrane (28). ANP Inhibits G Protein Activation

![ANP stimulates the translocation of RGS3 (A) and RGS4 (B) from the cytosol to the cell membrane.](image)

Cells were pre-labeled with $[^{35}\text{S}]$methionine as described and then incubated for 2 min with 10 nM peptides. Membrane and cytoplasmic fractions were prepared, total protein was normalized by BCA assay, and RGS proteins were immunoprecipitated and then separated by SDS-PAGE. Membrane RGS proteins are shown in the upper panel and the cytoplasmic content is shown in the lower panel. The ANP effects were reversed by ET-3, or an inactivator of the natriuretic peptide GC receptor, HS-142–1. The studies were repeated three times to create the bar graphs, which represent the mean ± S.E., and a representative study is shown. *, $p < 0.05$ for control versus ANP; +, $p < 0.05$ for ANP versus ANP plus ET-3 or HS-142–1.
membrane (protein at the membrane (above) and cytosol (below) is shown in Fig. 2); the amount of RGS at the membrane increased, whereas the amount of RGS in the cytoplasm decreased. If the membrane to cytoplasm RGS density ratio was arbitrarily designated as 1 in the absence of ANP, this peptide increased the ratio to approximately 3 for each RGS protein. Interestingly, ET-3 blocked the ability of ANP to stimulate RGS translocation (Fig. 2, A and B, lanes 4 versus 6). This suggests that ligation of GPCR by ET-3 inhibits a mechanism that could lead to a more rapid or stronger inactivation of G proteins (also see below). In contrast to Fig. 1, only ANP but not C-ANP 4–23 stimulated RGS translocation (lanes 3 versus 4). The translocation of RGS is directly attributed to ANP activation of the GC-A receptor (29), because only ANP and not C-ANP 4–23 caused this effect. Further, these actions of ANP were blocked by HS-142–1 (lanes 7 versus 4), a specific inhibitor of natriuretic peptide receptor guanylate cyclase activation (30).

ANP Stimulates RGS Association with Goq and Giq in the Membrane—We then found that ANP significantly stimulated in vivo the increased association of RGS 3 with Goq (Fig. 3A) and Giq (Fig. 3B). The natriuretic peptide also stimulated the association of RGS4 with both G protein subunits (Fig. 3, C and D). These effects were significantly reversed by HS-142–1, supporting the generation of cGMP as effector. Again, ET-3 blocked the association of the RGS with the two Ga subunits, and C-ANP 4–23 had no effect. These data support the idea that increasing association of RGS with Ga contributes to the ANP inhibition of G protein activation.

ANP Stimulates GTPase Activity—Increasing translocation and association of RGS with Goq and Giq is predicted to lead to an increased GTPase activity and down-regulation of G protein activity, based upon results from reconstituted systems (15–19). Whole astrocytes were incubated without or with ANP, ET, or both peptides for 2 min, and membranes were prepared from cells subjected to each condition. In parallel, membranes were made from cells that expressed an active catalytic subunit of PKG. The membranes from a given condition were then re-exposed to that condition (medium alone (control) or ANP, ET, or both peptides). Membranes from the catalytic subunit PKG-derived cells were exposed to medium alone, and a time course of GTPase activity was then carried out in all

![Fig. 3. ANP stimulates the increased association of RGS3 (A and B) or RGS4 (C and D) with both Goq (A and C) and Giq (B and D) proteins.](image-url)
membranes. As seen in Fig. 4, membranes from cells incubated with ANP alone responded to subsequent ANP exposure with a significant increased GTPase compared with control membranes exposed to medium alone; this was seen as early as 1–2 min after initiating the GTPase reaction. Membranes from cells expressing active PKG exhibited comparably stimulated GTPase activity when exposed to medium alone. This is consistent with the ability of ANP or catalytic PKG to cause RGS translocation to the membrane.

ET-3, as might be expected, potently stimulated a near 4-fold increase in GTPase activity, which peaked at 5 min. When membranes from astrocytes that were exposed to ANP + ET-3 were subsequently co-exposed to ANP and ET-3, there was a marked acceleration of maximum GTPase activity in this setting, compared with membranes/treatment with ET-3 alone. Maximal activity of GTPase was now seen at 2 min. This novel action of ANP would result in the rapid down-regulation of G protein activation and subsequent downstream signaling; the latter is known to occur in response to ANP (31).

However, because we know that ET prevents ANP or PKG-stimulated RGS translocation at this time (Fig. 2), it is likely that there are two separate and important effects of ANP. The ability of ANP to shift the kinetics of GTPase activity in the setting of ET-3 is not because of increased translocation of RGS but could involve multiple complex mechanisms of ET and ANP action. A second action is seen in response to ANP alone or from the expression of PKG. The stimulation of GTPase above control shown in these two situations may result from the effects of the translocated RGS.

To support the latter notion, we also carried out a GTPase time course study in astrocyte membranes from cells that were never exposed to either peptide. Here, subsequent incubation of the naïve membranes with ANP alone did not increase GTPase activity, compared with medium-incubated naïve membranes (data not shown). This indicates that the previous action of ANP alone could have been because of the stimulation of RGS translocation to the membrane. Importantly, the action of ANP to accelerate GTPase in the setting of co-incubation with ET-3 was also seen in the membranes from the naïve cells. This supports the idea that this novel action of ANP is unrelated to the translocation of RGS.

Cross-talk between PKG and G Proteins through RGS—We postulated that the ANP effects shown occur when increased cGMP generated from GC-A activation stimulates PKG (reviewed in Ref. 32). As one effector of cGMP, PKG often exists in two forms (Types I and II) (33, 34). PKG-I is produced as two isoforms and has been identified both in the cytoplasm and associated with the cell membrane (35). Supporting the role of this kinase, KT5823, at a concentration (0.5 μM) that is known to specifically inhibit PKG activity (36), prevented the ANP-induced translocation of both RGS3 (Fig. 5A) and RGS4 (Fig. 5B). In contrast, KT5720, a specific inhibitor of protein kinase A (37), had no effect on these actions of ANP. We subsequently used a concentration of KT5720 equivalent to the concentration of KT5823, and there was no reversal of the ANP effect (data not shown). Although protein kinase A is a known target for cGMP in some cells (38), it apparently is not important for ANP-induced translocation of RGS proteins in astrocytes.

We also transfected the astrocytes with a constitutively active catalytic subunit of PKG I. The increased PKG activity stimulated the translocation of RGS3 and RGS4 to the cell membrane, which was blocked by KT5823 (Fig. 5C). In addition, 8-Br-cGMP comparably stimulated RGS3 and -4 translocation from the cytosol to the membrane compared with PKG (Fig. 5D). In both instances, ET-3 substantially prevented these actions, indicating several levels of regulation by this peptide. These results demonstrate a novel cross-talk between the two signal transduction systems and considering all the results, indicate that G protein activation can be down-regulated by PKG, at least in part through effects on RGS proteins.

Phosphorylation of RGS by PKG—We speculated that the ability of PKG to cause the translocation of RGS proteins was related to the ability of this kinase to stimulate the phosphorylation of RGS. To begin to address this, we showed that ANP promoted the increased phosphorylation of RGS in the astrocyte and that this was prevented in vivo by KT5823 (Fig. 6A). Consistently, ET blocked the phosphorylation of RGS in response to ANP. To further support the role of PKG, we examined RGS phosphorylation in astrocytes expressing the active catalytic subunit of PKG I in the absence of ANP. This kinase clearly resulted in the phosphorylation of RGS3 and -4, partially reversed by KT5823 and ET (Fig. 6B). Because translocation of RGS occurs in a cGMP/PKG-dependent fashion, these additional findings are consistent with the hypothesis that phosphorylation and translocation of RGS occur in response to PKG and are linked.

DISCUSSION

The inhibition of growth factor-induced signaling is an important mechanism by which antigrowth factors limit cell proliferation. This interaction could occur proximally or distally (39) in signal transduction pathways. We now show that the anti-mitogen, ANP, acts very proximally to inhibit ET-3-induced Go, and Go, activation.

How does ANP modulate G protein activity? We found that there are several mechanisms potentially involved mediated by either the NPCR or the GC-A receptor. One mechanism likely involves the novel ability of ANP to stimulate the translocation of RGS3 and RGS4 to the cell membrane. It had been previously postulated that membrane translocation is necessary for the ability of RGS to inactivate G proteins (28). The increased
Fig. 5. Regulation of RGS translocation by PKG. The translocation of RGS 3 (A) and RGS 4 (B) to the cell membrane in response to ANP was inhibited by KT8823 but not KT5720. Equal total protein aliquots of membranes from astrocytes incubated with ANP ± ET-3 or kinase
ANP Inhibits G Protein Activation

The ability of ANP to cause the translocation of RGS proteins shown here resulted from their association with Goq and Goi. Translocation and association of RGS3 and -4 with Go subunits could lead to augmented GTPase activity, based upon many studies that show enhanced GTPase activation results from the interaction of expressed RGS proteins with Go subunits (15–19). In fact, we have found that ANP (or catalytically active PKG) can stimulate membrane GTPase activity. Both RGS4 and RGS3 are known inactivators of Go and Gi (16, 40) and therefore may contribute to the inhibitory actions of ANP on signal transduction in the astrocyte. This may in turn contribute to the quiescent basal state of the astrocyte or limit activation of proliferation by growth factors (8). However, it is also possible that other RGS proteins may contribute. Because RGS4 is known to inhibit Goq-induced extracellular signal-regulated kinase activation (16, 41), regulation of this protein is particularly attractive to explain some of the antiproliferative actions of ANP in our model.

The ability of ANP to cause the translocation of RGS proteins to the membrane occurs as the result of cGMP generation, because HS-142–1 reversed these effects of the natriuretic peptide. The cGMP second messenger can activate several effectors, but the cross-talk between signal transduction systems demonstrated here largely results from the activation of PKG. Our finding that a specific inhibitor of PKG reversed the actions of ANP on RGS supports this. Furthermore, expressing an active catalytic subunit of PKG in the absence of ANP stimulated RGS translocation to the cell membrane, as did 8-Br-cGMP. We found that inhibition of protein kinase A did not alter the actions of ANP.

How does PKG modulate RGS function? RGS3 can be phosphorylated by protein kinase A in vitro,2 and it is speculated that this might be involved in the translocation of RGS to the membrane (28). We also know that RGS3 is phosphorylated in vivo by an undetermined kinase, based upon 32P cell-labeling studies.2 PKG may be such a kinase, because we show here in vivo that ANP can stimulate the phosphorylation of RGS3 and -4 reversed by the PKG inhibitor KT5823. Furthermore, an active PKG expressed in the absence of ANP, also caused the phosphorylation of RGS. Several target substrate sequences for PKG (RKX(S/T), where X is any amino acid) are found within RGS3 and RGS4, and we have recently found that PKG can directly phosphorylate both these proteins in vitro.3

In cells overexpressing RGS3, it has been recently shown that ET-1 can stimulate the translocation of this protein to the membrane (42). This could provide a mechanism by which ET eventually restores the G protein inactive state and is consistent with our data, which indicates that ET activates peak GTPase activity at 5 min. Through hastening the activation and subsequent decay of GoGTase activity, ANP inhibits G protein stimulation and may also contribute to resetting the receptor-G protein interaction at a quiescent level, awaiting the next stimulation by ET. It is likely that ANP accelerates GTPase activity in the setting of ET-3, by a mechanism apart from RGS translocation. This is because ET-3 prevented the ANP, cGMP, or PKG-induced translocation of RGS at 2 min. The exact mechanism of interaction is probably very complex. However, our novel observation that ANP accelerates GTPase activity in this setting is likely to be important in explaining the down-regulation of G protein-activated signal transduction. As additional mechanisms, G protein activation can be negatively modulated by cGMP and/or PKG through the phosphorylation of GPCR or Gi via PKG (43, 44) and may contribute to the overall effects of ANP.

In turn, growth factors can down-regulate cGMP production induced by natriuretic peptides (45), and this probably underlies the antagonism by ET of ANP actions on RGS shown here. Interestingly, our results indicate that ET-3 also blocks the ability of cGMP to stimulate RGS translocation, and the ability of active PKG to stimulate RGS translocation or phosphorylation. Thus, ET-3 seems to be capable of inhibiting several steps in the ANP-induced signal transduction pathway. These results support our additional proposal that down-regulation of the ANP-restraining mechanism might be necessary for full G protein activation by GPCR ligands. Acceleration of GTPase activity and inhibition of G protein activation by ANP could be important for the modulation of vascular smooth muscle vasomotor tone. In vascular smooth muscle and related cells, ET

**Fig. 6. Phosphorylation of RGS by PKG.** A. ANP stimulated the increased incorporation of [32P]ATP into RGS3 and RGS4, which was reversed by KT5823 or ET-3. Labeled astrocytes were incubated with ANP ± KT5823 or ET for 2 min and then assayed for phosphorylation of RGS, as described under “Materials and Methods.” B. expression of an active catalytic subunit of PKG-I, in the absence of ANP, also caused the phosphorylation of RGS3 and -4. Representative experiments shown here are repeated twice.

**Table 1.**

| Condition       | RGS3 | RGS4 |
|-----------------|------|------|
| Control         |      |      |
| ANP             |      |      |
| ANP + KT5823    |      |      |
| ANP + ET3       |      |      |

**Legend:**

- **A.** ANP induced translocation of RGS proteins shown here resulted in their association with Goq and Goi. Translocation and association of RGS3 and -4 with Go subunits could lead to augmented GTPase activity, based upon many studies that show enhanced GTPase activation results from the interaction of expressed RGS proteins with Go subunits (15–19). In fact, we have found that ANP (or catalytically active PKG) can stimulate membrane GTPase activity. Both RGS4 and RGS3 are known inactivators of Go and Gi (16, 40) and therefore may contribute to the inhibitory actions of ANP on signal transduction in the astrocyte. This may in turn contribute to the quiescent basal state of the astrocyte or limit activation of proliferation by growth factors (8). However, it is also possible that other RGS proteins may contribute. Because RGS4 is known to inhibit Goq-induced extracellular signal-regulated kinase activation (16, 41), regulation of this protein is particularly attractive to explain some of the antiproliferative actions of ANP in our model.

- **B.** expression of an active catalytic subunit of PKG-I, in the absence of ANP, also caused the phosphorylation of RGS3 and -4. Representative experiments shown here are repeated twice.

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1. A. Scheshonka and J. Kehrl, submitted for publication.
2. A. Pedram, M. Razandi, and E. R. Levin, unpublished observations.
rapidly stimulates whereas ANP inhibits calcium flux and other downstream signaling (46, 47), perhaps resulting from similar G protein interactions.

In summary, we have identified that ANP can down-regulate G protein activation in primary cells. Increased GTPase activity in response to ANP alone or active PKG expression is likely to result from the demonstrated novel cross-talk between PKG and RGS proteins. ANP also accelerates GTPase activation in a native state providing a mechanism to understand the previously demonstrated down-regulation of signaling in response to GPCR ligand (8, 31). The model can be used to discern the details of GRS function and G protein activation/deactivation in a native state cell. Further understanding of these interactions could lead to therapeutic intervention in unwanted proliferation, because several pharmacologic agents that stimulate PKG activation are in clinical use for other purposes.

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