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Direct Evidence for Functional Coupling of the Vasoactive Intestinal Peptide Receptor to G_{i3} in Native Lung Membranes

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SUMMARY

Although vasoactive intestinal peptide (VIP) exerts many of its effects through stimulation of adenylyl cyclase, there is increasing evidence that other signaling pathways may contribute to its action. The role of inhibitory G proteins (G_i) in VIP-mediated signaling in the lung was assessed by a combination of equilibrium-binding and covalent cross-linking studies. Pertussis toxin treatment of rat lung membranes reduced the high affinity binding of ^{125}I-VIP, implicating a member of the G_i family in signaling from the VIP receptor. The particular G protein involved was identified as G_{i3} through capture of a VIP/receptor/G_{i3} ternary complex by covalent cross-linking. There was a progressive rise with increasing VIP concentration in formation of the complex reported by the cross-linking strategy. Guanine nucleotides and an anti-$G_{\alpha i3}$ antiserum suppressed formation of the VIP/receptor/G_{i3} ternary complex, demonstrating its functional nature in native lung membranes. Inhibition of high affinity ^{125}I-VIP binding by the anti-$G_{\alpha i3}$ antiserum verified this functionality. Taken together, these data suggest that receptor/G_{i3} coupling makes a significant contribution to VIP-mediated signaling in the lung and illustrate the value of covalent cross-linking as a strategy to define receptor/G protein complexes that arise under conditions in which the stoichiometry and microdomains of the native cell membrane are preserved.

VIP is widely distributed in the peripheral and central nervous systems, where it acts as a neurotransmitter and neuromodulator (1). In lung, VIP is a potent smooth muscle relaxant, inducing both bronchodilation and vasodilation (1, 2). It has also been implicated in the immune response and may modulate alveolar macrophage function (3). Recent studies have demonstrated the therapeutic potential of VIP analogs in asthma (2, 4). Two subtypes of VIP receptor have recently been identified by cloning (5, 6); both subtypes are predicted to be members of the G protein-coupled receptor superfamily. There is considerable evidence that VIP exerts many of its effects through stimulation of adenylyl cyclase (1, 2, 7). Nevertheless, there are also data to suggest that the VIP receptor in some tissues may couple to other signaling pathways, including inhibition of adenylyl cyclase (8), stimulation of phospholipase C (9), and stimulation of nitric oxide synthase (10).

We recently established a strategy to define interactions between receptors and heterotrimeric G proteins in the native membrane milieu; functional interactions are detected through capture by covalent cross-linking of the ligand/receptor/G protein ternary complex that initiates the signal transduction cascade (11). We previously used this strategy to visualize the VIP/receptor/G_s ternary complex that mediates adenylyl cyclase stimulation (11, 12). Here, we demonstrate the involvement of a pertussis toxin-sensitive G protein in signal transduction from the VIP receptors in lung tissues and apply the cross-linking approach to identify the specific G protein involved. This study demonstrates for the first time that VIP receptors are coupled functionally to G_{i3}. The same approach can be used to define coupling between other receptors and G proteins in the native membrane milieu.

Experimental Procedures

Materials. ^{125}I-VIP (2200 Ci/mmol) was purchased from New England Nuclear Research Products (Boston, MA), and nonradioactive VIP was from Peninsula Laboratories (Belmont, CA). Pertussis toxin A protomer and B oligomer were obtained from Calbiochem (San Diego, CA). The homobifunctional cross-linker EGS was purchased from Pierce Chemical (Rockford, IL); it has two *N*-hydroxysuccinimide ester groups separated by a spacer of 1.61 nm and forms cross-links between primary amino groups (especially ε-amino groups on lysine) (13). GTP, Gpp(NH)p, and guanosine-5′-*O*-(thiotriphosphate) were obtained from Sigma Chemical (St. Louis, MO), and SDS was from Serva (Garden City Park, NY). Protein molecular mass markers and nitrocellulose membranes (0.45-μm pores) were obtained from BioRad (Richmond, CA). Horseradish peroxidase-con-

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ABBREVIATIONS: VIP, vasoactive intestinal peptide; EGS, ethylene glycol-bis(succinimidylsuccinate); Gpp(NH)p, guanylyl 5′-imidodiphosphate; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate.

jugated donkey anti-rabbit second antibody and the enhanced chemiluminescence Western blotting detection system were from Amersham (Arlington Heights, IL). All other materials were of the highest purity available.

Polyclonal rabbit anti-G protein antisera and recombinant G protein α subunit standards were purchased from Calbiochem. The antisera were raised against synthetic peptides that are unique to specific G protein α subunits (carboxyl-terminal decapeptides) or conserved in the G protein β subunits (Lys127-to-Leu139) (14–17). Most of these antisera are specific for the appropriate G protein subunit (Fig. 1, A–E). The anti-$G_{\alpha i1/2}$ antiserum, however, recognizes both $G_{\alpha i1}$ and $G_{\alpha i2}$ (Fig. 1D) because they share the same carboxyl-terminal sequence. Two different anti-$G_{\alpha i3}$ antisera (both from Calbiochem) were used; these antisera were obtained from different rabbits that were injected with the same immunogen (carboxyl-terminal decapeptide of $G_{\alpha i3}$), but their properties differ. Antiserum I has been affinity purified (using the carboxyl-terminal $G_{\alpha i3}$ decapeptide) and reacts specifically with $G_{\alpha i3}$ (Fig. 1A), but it does not perturb VIP/receptor/G_{i3} coupling[2]; antiserum II inhibits such coupling (Fig. 5B) but also cross-reacts weakly with $G_{\alpha o}$ (Fig. 1B).

Isolation of rat lung and brain plasma membranes. A fraction enriched in plasma membranes was isolated from rat lung by differential centrifugation after homogenization in the presence of a cocktail of protease inhibitors, as described previously (11). Rat brain plasma membranes were isolated by an analogous procedure (12).

Pertussis toxin treatment. Rat lung membranes (100 μg of protein) were incubated at 30° with 0.25 μg of pertussis toxin A protomer or 1 μg of pertussis toxin B oligomer (inactive) in the presence of 20 μM of NAD in 100 μl of buffer A (150 mM $NaPO_4$, pH 7.4, 10 mM thymidine, 0.5 mM ATP). Excess reactants were removed by centrifugation and resuspension before assay of ^{125}I-VIP binding.

Preliminary experiments established the time course for pertussis toxin-mediated ADP-ribosylation of the G protein α subunits in lung. The anti-$G_{\alpha i1/2}$ and anti-$G_{\alpha i3}$ antisera used in this study recognize epitopes that encompass the cysteine residue undergoing ADP-ribosylation in each G protein α subunit. Immunoblots with these antisera indicate that the ADP-ribosylation reaction can be monitored through a decrease in antibody reactivity. Such a decrease has been observed previously with some anti-G_α antibodies (18) but not with others (19). This approach showed that ADP-ribosylation of the G protein α subunits in lung was essentially complete after a 15-min reaction; prolonged treatment (2 hr) resulted in little additional reduction in band intensity on the immunoblots (Fig. 2C).

Assay of high affinity ^{125}I-VIP binding. Rat lung membranes (100 μg of protein) were incubated to equilibrium (30 min at 37°) with 250 pM ^{125}I-VIP in buffer B (1 mM $MgSO_4$, 50 mM $NaPO_4$, pH 7.4). Nonspecific binding was evaluated in the presence of 200 nM nonradioactive VIP. Receptor-bound and free ^{125}I-VIP were separated by rapid filtration (20) or centrifugation. This single-point measurement of ^{125}I-VIP binding estimates the binding capacity of receptors in the high affinity state.

The saturation binding characteristics of rat lung VIP receptors were assessed using a range of concentrations of ^{125}I-VIP (3–300 pM) chosen to define the high affinity binding component that reflects receptor/G protein coupling. Equilibrium-binding data were analyzed using the LIGAND computer program (21).

Cross-linking of VIP to its receptor and the receptor to associated G proteins. Rat lung membranes (500 μg of protein) were incubated for 30 min at 23° with 250 pM ^{125}I-VIP or 10 nM nonradioactive VIP (as indicated) in buffer B. Free ^{125}I-VIP or VIP was removed by centrifugation (12,300 × *g* for 5 min) and resuspension of the membranes in buffer B. Covalent cross-linking of the VIP/receptor/G protein ternary complexes was accomplished by incubation (30 min at 23°) with 5 mM EGS. The cross-linking reaction was quenched with 20 mM glycine.

[2] N. L. Diehl and S. M. Shreeve, unpublished observations.

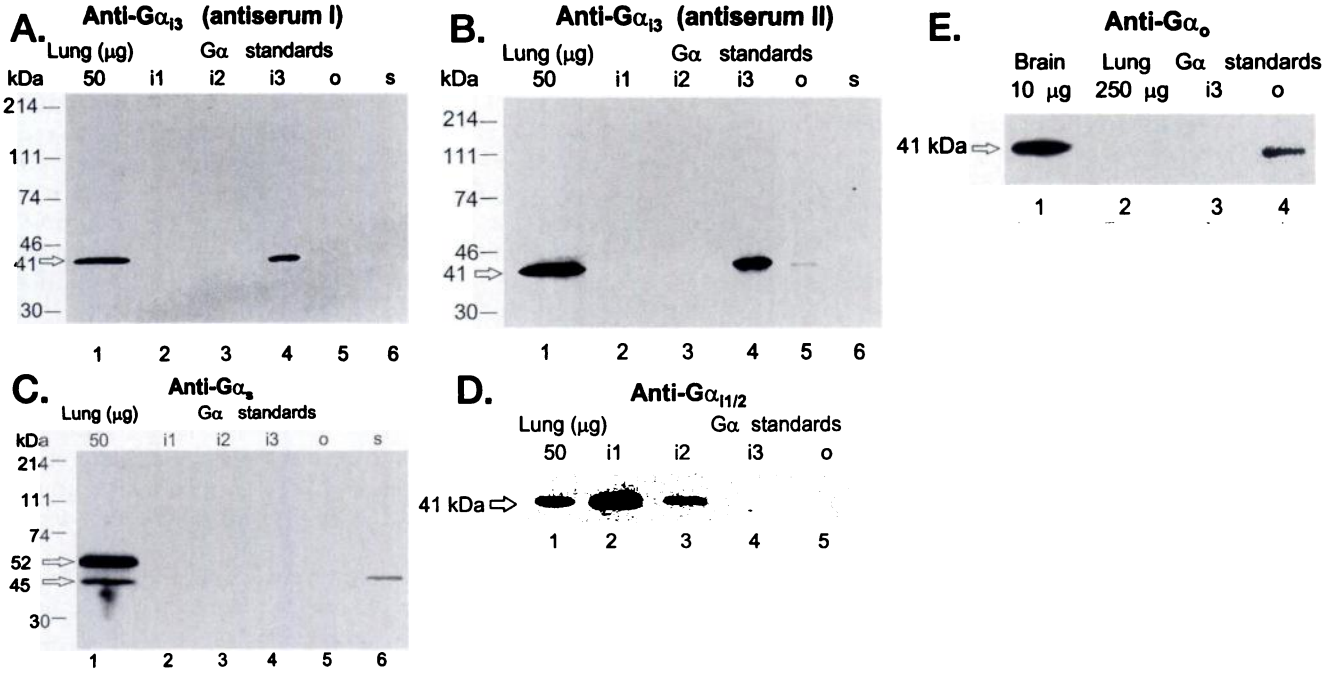

Fig. 1. Assessment of specificity for anti-G_α antisera on immunoblots. A–D, Rat lung membranes (50 μg of protein, *lane 1*), without VIP treatment or cross-linking, and G_α standards (*lanes 2–6*) were analyzed by 8% SDS-PAGE and immunoblotting. The standards comprised recombinant $G_{\alpha i1}$, $G_{\alpha i2}$, $G_{\alpha i3}$, $G_{\alpha o}$, and $G_{\alpha s}$ (short form); nominally equal amounts were loaded. Immunoblots were probed with the antisera against $G_{\alpha i3}$ (antiserum I, *A*; antiserum II, *B*), $G_{\alpha s}$ (*C*), and $G_{\alpha i1/2}$ (*D*). *Open arrows*, G protein α subunits detected in lung. These control immunoblots verify that all antisera except the anti-$G_{\alpha i3}$ antiserum II are specific for the appropriate G protein α subunit(s). E, Absence of detectable $G_{\alpha o}$ in rat lung. Rat brain membranes (10 μg of protein, *lane 1*) and lung membranes (250 μg of protein, *lane 2*), without VIP treatment or cross-linking, and recombinant G_α standards ($G_{\alpha i3}$, *lane 3*; $G_{\alpha o}$, *lane 4*) were analyzed by 8% SDS-PAGE and immunoblotting with anti-$G_{\alpha o}$ antiserum. These data demonstrate that the cross-reactivity of anti-$G_{\alpha i3}$ antiserum II toward $G_{\alpha o}$ is irrelevant in this study.

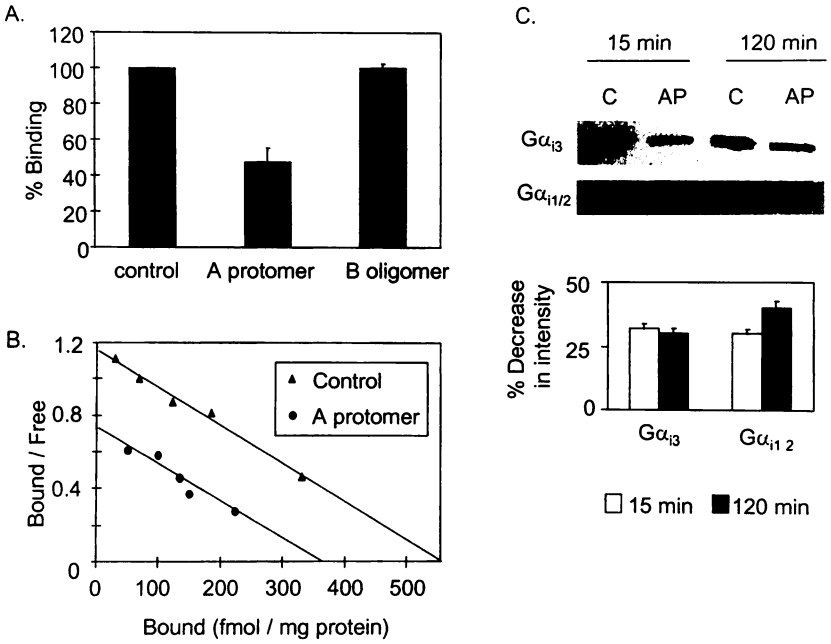

Fig. 2. Effect of pertussis toxin treatment of rat lung membranes on high affinity ^{125}I-VIP binding. A, Lung membranes were pretreated (15 min at 30°) without toxin (control) or with either pertussis toxin A protomer or B oligomer (as indicated). Equilibrium binding of 250 pM ^{125}I-VIP was then assayed. Specific binding data (mean ± standard error, n = 6) are presented as a percentage of the control. B, Lung membranes were pretreated (15 min) without (▲) or with (●) pertussis toxin A protomer. Equilibrium binding was assayed using 3–300 pM ^{125}I-VIP. Specific binding data from a typical one of three studies are presented as a Scatchard plot. Analysis of these data yielded K_d estimates of 48 and 49 pM for the control and toxin-treated membranes, respectively; binding capacity was estimated as 560 and 360 fmol/mg of protein, respectively. C, Lung membranes were pretreated (15 or 120 min, as indicated) without (control, *C*) or with (*AP*) pertussis toxin A protomer. Samples were analyzed by SDS-PAGE, transferred to nitrocellulose, and immunoblotted with antiserum against $G_{\alpha i3}$ (antiserum II) or $G_{\alpha i1/2}$ (as indicated). *Top*, immunoblot data from a typical study. *Bottom*, percentage reduction in intensity (relative to control) of each $G_{\alpha i}$ band after pertussis toxin treatment (mean ± standard error, n = 2), derived by scanning densitometry. These immunoblots demonstrate that ADP-ribosylation of $G_{\alpha i3}$ is complete and that ADP-ribosylation of the other $G_{\alpha i}$ is nearly complete after a 15-min treatment with the pertussis toxin A protomer.

SDS-PAGE analysis. Samples were solubilized (20 min at 23°) in SDS reduced sample buffer (10% glycerol, 1% 2-mercaptoethanol, 2% SDS, 0.001% bromophenol blue, 62.5 mM Tris·HCl, pH 6.8) and analyzed by discontinuous SDS-PAGE (8% gels) (22). Autoradiography (for 2–14 days) was performed using X-ray film (Amersham Hyperfilm-ECL or Kodak XAR-5).

Immunoblotting. Proteins were transferred from the polyacrylamide gel to nitrocellulose (11, 23), and G protein subunits were identified by immunoblotting with specific anti-G protein antisera (used at 1:1000 dilution) (11). The primary antibodies were detected with a horseradish peroxidase-conjugated second antibody and visualized by chemiluminescence, and relative band intensities were quantified by scanning densitometry (11).

Cross-linked samples to be probed with anti-G_β antiserum were immunoprecipitated with an anti-$G_{\alpha common}$ antiserum (Calbiochem) before electrophoresis because the anti-G_β antiserum otherwise tended to cross-react excessively with other membrane proteins on the immunoblot (11). Immunoprecipitation was effected according to the method of Laugwitz *et al.* (24), after membrane solubilization and protein denaturation with 4% SDS, 1% Nonidet P-40, and 1% deoxycholate.

Results and Discussion

Because the results of previous studies have suggested that VIP may inhibit adenylyl cyclase in some cells (8), we investigated whether the VIP receptors in lung are functionally associated with a member of the G_i family. Pertussis toxin selectively ADP-ribosylates the α subunit of G proteins in this family (which also includes G_o and transducin) and prevents their interaction with receptors (25). High affinity ^{125}I-VIP binding declined substantially on treatment of rat lung membranes with pertussis toxin A protomer ($p < 0.001$, analysis of variance with Bonferroni's t test; Fig. 2A). In contrast, treatment with the inactive B oligomer of the toxin had no effect ($p > 0.5$). Saturation-binding studies indicated that the effect of the pertussis toxin A protomer was to reduce the binding capacity of the high affinity sites ($p < 0.05$, paired t test; Fig. 2B). Binding affinity, in contrast, was unaffected ($p > 0.1$). This is the pattern to be anticipated for a receptor that is coupled to two G proteins, only one of which is sensitive to pertussis toxin; it reflects the elimination of some receptor/G protein complexes (those involving a member of the G_i family) but the retention of others (those involving G_s). Immunoblotting data confirmed that ADP-ribosylation of $G_{\alpha i3}$ was complete (and that of other $G_{\alpha i}$ was nearly complete) under the conditions used (Fig. 2C). This result therefore implies that at least some VIP receptors in rat lung are coupled to an inhibitory G protein; more specifically, it implies coupling to G_{i1}, G_{i2}, or G_{i3} because the lung does not contain G_o (Fig. 1E).

The cross-linking strategy that we developed to capture functional ligand/receptor/G protein ternary complexes (11, 12) was used to identify the specific member of the G_i family that is involved in VIP receptor signaling in lung. VIP/receptor/G protein ternary complexes in rat lung plasma membranes were covalently cross-linked and probed with antisera directed against specific G protein α subunits.[3] In confirma-

[3] One limitation of the cross-linking approach is that it does not necessarily permit quantitative assessment of the relative prevalence of different complexes on the basis of band intensities on an immunoblot. Lysine residues are

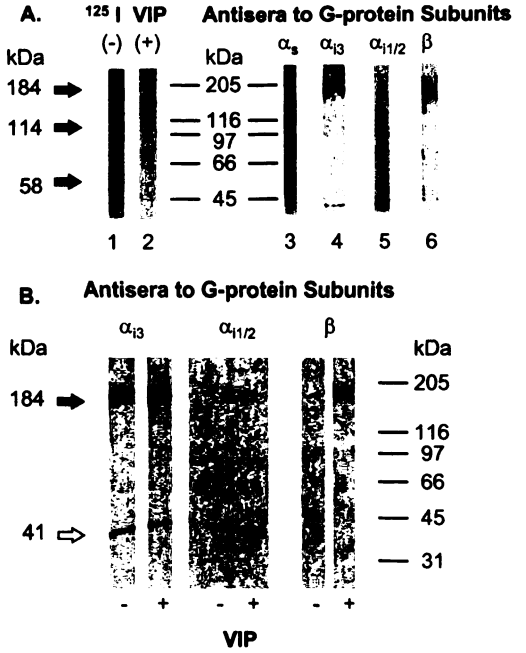

Fig. 3. Identification of G protein subunits in captured VIP/receptor/G protein ternary complexes in rat lung. A, Radiolabeled VIP/receptor/G protein ternary complexes were captured by incubation of lung membranes with 250 pM ^{125}I-VIP (−; *lane 1*) and covalent cross-linking with 5 mM EGS. Nonspecific cross-linking was assessed in a parallel sample that included both ^{125}I-VIP and 100 nM nonradioactive VIP (+; *lane 2*). Analogous unlabeled ternary complexes were captured by incubation with 10 nM nonradioactive VIP and cross-linking with EGS (*lanes 3–6*). Samples were analyzed by SDS-PAGE and either autoradiographed (*lanes 1* and *2*) or immunoblotted on nitrocellulose (*lanes 3–6*). Immunoblots were probed with antiserum against $G_{\alpha s}$ (*lane 3*), $G_{\alpha i3}$ (antiserum I, *lane 4*), $G_{\alpha i1/2}$ (*lane 5*), or G_β (*lane 6*). The sample probed with anti-G_β antiserum (*lane 6*) was immunoprecipitated with an anti-$G_{\alpha common}$ antiserum before electrophoresis. *Solid arrows*, specifically labeled proteins. The 58-kDa band represents the VIP/receptor complex, and the 184-kDa band represents the VIP/receptor/G protein ternary complexes (11); whether the G protein γ subunit is included in the latter band remains to be established. The 114-kDa band may arise from incomplete cross-linking of ternary complexes. Although a native $G_{\alpha s}$ band is not visible on the anti-$G_{\alpha s}$ immunoblot (*lane 3*), such a band was detected on prolonged exposure. The greater prominence of native $G_{\alpha i3}$ and $G_{\alpha i1/2}$ bands (*lanes 4* and *5*, respectively) may reflect the greater prevalence of the inhibitory G proteins. B, Receptor/G protein complexes were captured by cross-linking with EGS after incubation of lung membranes in the absence (−) or presence (+) of 10 nM nonradioactive VIP. Samples were analyzed by SDS-PAGE, transferred to nitrocellulose, and then immunoblotted with antiserum against $G_{\alpha i3}$ (antiserum I), $G_{\alpha i1/2}$, or G_β, as indicated. The samples probed with anti-G_β antiserum were immunoprecipitated with an anti-$G_{\alpha common}$ antiserum before electrophoresis. *Solid arrow*, 184-kDa receptor/G protein complex; *open arrow*, native α subunit (41 kDa) of the G protein. The native G protein β subunit is lost during the immunoprecipitation step and thus not observed on the immunoblot.

tion of our previous findings, the anti-$G_{\alpha s}$ antiserum detected two major broad bands of 114 and 184 kDa (Fig. 3A, *lane 3*). These bands comigrated with bands visualized by autoradiography after cross-linking with ^{125}I-VIP (*lane 1*); we have shown previously that the bands detected by this antiserum compose a VIP/receptor/$G_{\alpha s}$ complex and an intact VIP/receptor/G_s ternary complex, respectively (11). Comigration of the 184-kDa band with the only band recognized by the anti-G_β antiserum (*lane 6*) confirmed the nature of these complexes. It is unclear, however, whether the 114-kDa moiety represents an incompletely cross-linked ternary complex or a true intermediate in G protein signaling (26).

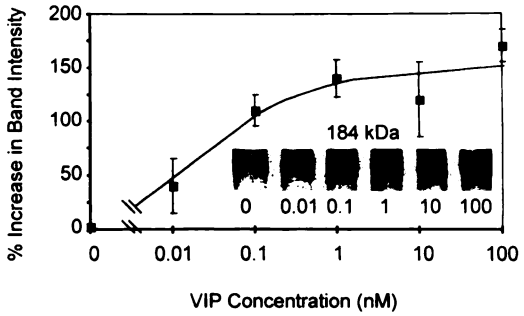

Fig. 4. Dose-response characteristics for coupling of the VIP receptor to G_{i3} in rat lung. The extent of VIP/receptor/G_{i3} ternary complex formation was assessed with increasing VIP concentrations. Rat lung membranes were incubated without or with VIP (0.01–100 nM). Cross-linking was effected with EGS. Samples were analyzed by 8% SDS-PAGE, transferred to nitrocellulose, and immunoblotted with antiserum against $G_{\alpha i3}$ (antiserum I). *Graph*, relative intensities (mean ± standard error, $n = 3$) for the captured VIP/receptor/G_{i3} complex (184-kDa band) derived by scanning densitometry; *inset*, original immunoblot data from a typical study.

The anti-$G_{\alpha i3}$ antiserum detected a similar broad band of 184 kDa (Fig. 3A, *lane 4*) together with native $G_{\alpha i3}$ (41 kDa). Another antiserum against $G_{\alpha i3}$ (antiserum II) revealed the same pattern (data not shown). Analogy with the anti-$G_{\alpha s}$ data implies that the top band represents a VIP/receptor/G_{i3} ternary complex.[4] The strength of the anti-$G_{\alpha i3}$ immunoblot signal for the 184-kDa band increased substantially when cross-linking was conducted in the presence of VIP, and there was a concomitant decrease in signal for the 41-kDa band that represents native $G_{\alpha i3}$ (Fig. 3B). VIP also promoted a marked rise in the anti-G_β immunoblot signal for the 184-kDa band. These changes together provide evidence of VIP-mediated receptor/G_{i3} coupling. In contrast to these observations with the anti-$G_{\alpha i3}$ antisera, the antiserum directed against $G_{\alpha i1/2}$ did not detect any higher molecular mass bands (Fig. 3A, *lane 5*). Furthermore, cross-linking in the presence of VIP did not result in a detectable diminution in signal for the native $G_{\alpha i1/2}$ band (Fig. 3B). Control immunoblots using recombinant G protein α subunits (Fig. 1) confirmed the specificity of the various anti-G_α antisera; in particular, the primary antiserum against $G_{\alpha i3}$ (antiserum I) did not cross-react with $G_{\alpha s}$ (or with any other α subunit) (Fig. 1A). These findings imply that the VIP receptors are coupled to G_{i3} as well as to G_s in the native microenvironment of the lung membrane; there is no evidence of coupling to G_{i1} or G_{i2}.

present in the epitopes for the anti-$G_{\alpha i1/3}$, anti-$G_{\alpha i3}$, and anti-G_β antisera. Cross-linking of (or monofunctional reaction with) these lysine residues (within a single G protein subunit, between two subunits, or between G protein and receptor) probably perturbs the epitope of the antibody and impairs detection. Consequently, it may be inappropriate to compare the intensities of immunoblot bands that represent different moieties (eg., free G_α and VIP/receptor/G protein complex). Nevertheless, a comparison that is restricted to a single cross-linked moiety (e.g., the ternary complex) under different conditions is meaningful because the pattern of cross-links in this moiety, and therefore antibody detection efficiency, is likely to be consistent under all conditions.

[4] Although neither anti-$G_{\alpha i3}$ antiserum reveals a band at 114 kDa, this should not be regarded as definitive evidence to rule out the existence of a VIP/receptor/$G_{\alpha i3}$ moiety. Such a complex might be undetectable on immunoblots as a result of blockade of the epitopes for both antibodies by cross-links to Lys345 or Lys349 within the carboxyl-terminal $G_{\alpha i3}$ decapeptide. The corresponding $G_{\alpha s}$ decapeptide (Arg385-to-Leu394), in contrast, has no lysine residues (27). The 184-kDa VIP/receptor/G_{i3} moiety may arise through cross-links that do not involve the carboxyl-terminal region of the G protein α subunit.

If coupling to G_{i3} plays a significant role in VIP receptor signaling in lung, then the extent of such coupling should be dose dependent. Formation of the VIP/receptor/G_{i3} ternary complex was thus evaluated at different VIP concentrations by the cross-linking approach (Fig. 4). The antiserum against $G_{\alpha i3}$ detected a weak band at 184 kDa in the absence of VIP (Figs. 3B and 4), possibly indicating precoupling of a receptor/G_{i3} complex. A progressive rise in the intensity of this band occurred with increasing VIP concentration (Fig. 4), reaching a plateau at 1 nM VIP. The EC_{50} (0.01–0.1 nM) for detection of this band corresponds well with K_d values for VIP/receptor binding (Fig. 2B). It is also comparable to previous EC_{50} estimates for VIP-induced receptor/G_s coupling (based on an assay of adenylyl cyclase stimulation) (5, 6). These dose-response data establish that the increase in the cross-linked complex arises from VIP-mediated receptor/G_{i3} coupling. The increment in the 184-kDa band detected by the antiserum must represent a true complex between the VIP receptor and G_{i3}; it cannot merely reflect random collisions between these moieties.

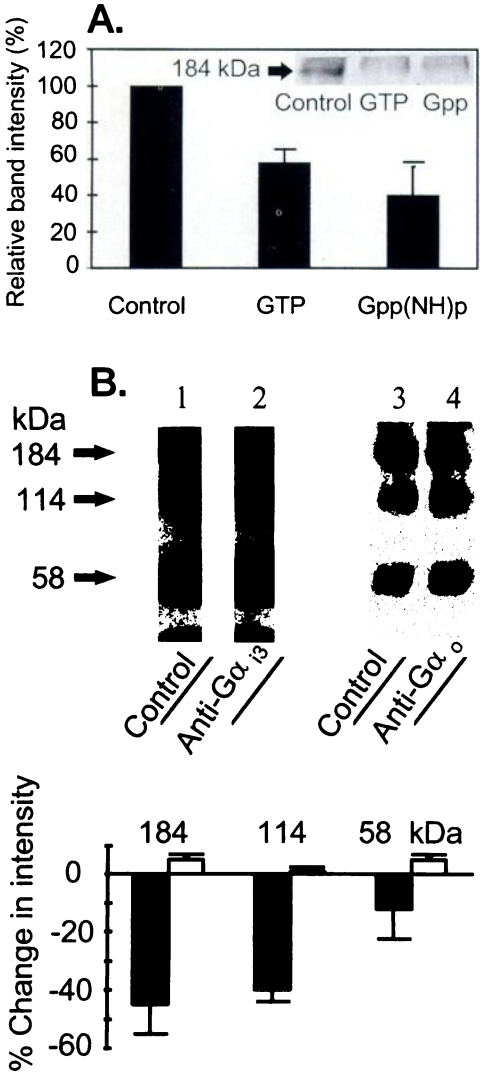

Fig. 5. Demonstration of the functional nature of the VIP/receptor/G_{i3} complex in rat lung. A, Influence of GTP analogs on formation of the VIP/receptor/G_{i3} ternary complex. Nonradioactive VIP (250 pM) was cross-linked to its receptors in lung by EGS in the absence (control) or presence of 1 mM GTP or Gpp(NH)p. Samples were analyzed by 8% SDS-PAGE and immunoblotted with antiserum against $G_{\alpha i3}$ (antiserum I). *Histogram*, intensities (relative to control) for the 184-kDa band (mean ± standard error, n = 2–4) derived by scanning densitometry. *Inset*, original immunoblot data from a typical study [*Gpp*, Gpp(NH)p]. B, Effect of anti-G_α antibodies on formation of VIP/receptor/G protein ternary complexes. Rat lung membranes (500 μg of protein) were preincubated (3 hr at 4°) in the absence (control; *lanes 1* and *3*) or presence of a 1:50 dilution of antiserum against $G_{\alpha i3}$ (antiserum II; *lane 2*) or $G_{\alpha o}$ (*lane 4*) in buffer C (150 mM NaCl, 20 mM Tris·HCl, pH 7.5, 10 mM $MgCl_2$). ^{125}I-VIP was subsequently cross-linked, and the captured ternary complexes were analyzed by SDS-PAGE and autoradiography. *Top*, autoradiographs from a typical study. *Bottom*, percentage change in intensity of each cross-linked band (mean ± standard error, n = 2) on preincubation with antiserum against $G_{\alpha i3}$ (*solid bars*) or $G_{\alpha o}$ (*open bars*). The modest decline in labeling of the 58-kDa band (VIP/receptor complex) that accompanies the reduction in the higher molecular mass bands is presumed to reflect the lower affinity of the receptor for VIP when it is not associated with G_{i3}.

The functional nature of coupling between the VIP receptor and G_{i3} was evaluated through several approaches. First, GTP and Gpp(NH)p suppressed formation of the cross-linked VIP/receptor/G_{i3} ternary complex in rat lung membranes ($p < 0.01$ for each; Fig. 5A), demonstrating its functionality. Guanosine-5′-*O*-(thiotriphosphate) also inhibited ternary complex formation (data not shown). The nonequilibrium nature of the cross-linking approach probably explains the finding that high concentrations of guanine nucleotides are required to inhibit ternary complex formation; cross-linking of any ternary complexes inevitably drives the reaction toward the formation of further complexes. Similar guanine nucleotide concentrations are required to prevent cross-linking of the VIP/receptor/G_s ternary complex (11). Second, an anti-$G_{\alpha i3}$ antiserum disrupted formation of the VIP/receptor/G protein ternary complex (detected using ^{125}I-VIP; Fig. 5B). Such disruption is expected for a functional ternary complex in view of the important contribution of the carboxyl-terminal region of the G protein α subunit (which contains the epitope for this antibody) to the receptor interaction site. This finding paralleled our previous observations with an anti-$G_{\alpha s}$ antiserum (11). In contrast, antiserum directed against $G_{\alpha o}$ had no effect on the ternary complex. Finally, increasing concentrations of anti-$G_{\alpha i3}$ antiserum progressively reduced the binding capacity of high affinity ^{125}I-VIP binding sites on rat lung membranes ($p < 0.001$; Fig. 6); anti-$G_{\alpha o}$ antiserum caused no such reduction ($p > 0.2$). As a diminution in high affinity binding sites implies uncoupling of the receptor from the G protein, these data provide confirmatory evidence of functional coupling to $G_{\alpha i3}$. The covalent cross-linking and equilibrium binding studies in combination provide definitive evidence that the observed coupling between the VIP receptor and G_{i3} is functional.

The present observations, taken together with our previous data (11), imply that, in rat lung, VIP can activate both G_{i3} and G_s. Prior studies by other investigators indicate that one of the cloned VIP receptor subtypes (VIP_1) is abundant in rat lung (5), whereas the other subtype (VIP_2) has not been reported in lung (6, 28). Each of the cloned VIP receptor subtypes stimulates adenylyl cyclase when expressed in COS cells (5, 6). These observations and our findings together suggest that one subtype of VIP receptor (probably the VIP_1 subtype) may be coupled to both G_{i3} and G_s in lung. Further studies, however, are required to establish this definitively and to determine whether the two signaling pathways coexist in the same cell. The primary effector for signaling mediated by G_{i3} remains uncertain, as does its role in the lung. Puta-

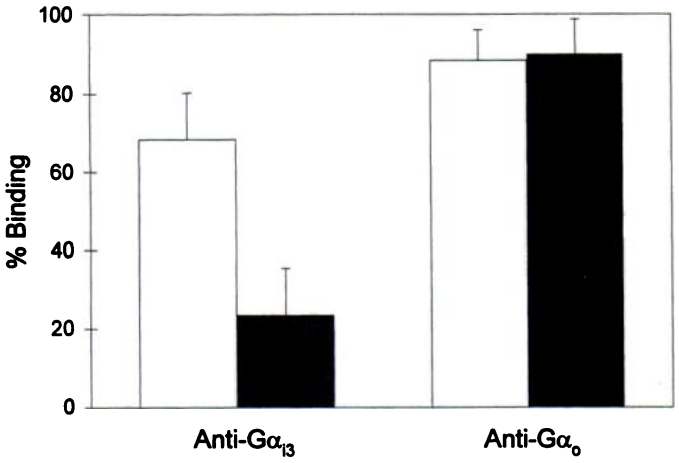

Fig. 6. Influence of anti-G_α antisera on high affinity ^{125}I-VIP binding to rat lung membranes. The binding capacity of the high affinity sites was assessed by incubation of lung membranes to equilibrium with 250 pM ^{125}I-VIP in the absence or the presence of a 1:10,000 (*open bars*) or 1:1,000 (*solid bars*) dilution of antiserum against $G_{\alpha i3}$ (antiserum II) or $G_{\alpha o}$. Specific binding data (mean ± standard error, n = 3–5) are expressed as a percentage of that in the absence of either antiserum.

tive functions for G_{i3} include stimulation of phospholipase C-β_3 (29) and inhibition of adenylyl cyclase (30). Coupling of a receptor to both G_{i3} and G_s (in the same cell), however, would be counterproductive if the sole function of G_{i3} were to inhibit adenylyl cyclase. An intriguing possibility is that $\beta\gamma$ subunits released from G_{i3} might act synergistically with $G_{\alpha s}$ to stimulate adenylyl cyclase; such synergism has been reported for the type II and IV isoforms of adenylyl cyclase (31–33). Coupling to both G_s and G_i has also been reported in some previous investigations of G protein-coupled receptors, including studies of α_2- (34) and β- (35) adrenergic receptors. Such investigations, however, have involved an imbalance in stoichiometry between receptors and G proteins due to overexpression of a receptor or deficiency in a G protein.

Several methods have been developed to examine receptor/G protein coupling: these include overexpression of receptors in foreign cells, reconstitution of purified components in liposomes, and immunoprecipitation of detergent-solubilized receptor/G protein complexes (36–39). Such approaches have shed invaluable light on potential receptor/G protein interactions. The present cross-linking strategy, however, offers significant advantages over previous approaches. In particular, this strategy is designed to assay receptor/G protein coupling in the native cell membrane milieu; it does not require nonphysiological quantities or an artificial stoichiometry of receptors and G proteins in an unnatural microenvironment.

In conclusion, in the current study, we identified a novel signal transduction pathway for VIP in the lung. The VIP receptor couples functionally to G_{i3}; this interaction complements the coupling to G_s, and both signaling pathways arise when the native receptor and G proteins are present at their normal stoichiometry. Furthermore, the study demonstrates the value of covalent cross-linking as an effective strategy for capturing and visualizing functional receptor/G protein interactions that occur in the native cell membrane.

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