Insect cell expression systems are used to characterize signaling components such as G protein-coupled receptors. As such, one must know whether endogenous G proteins couple to non-native receptors. We examined G protein linkages after infection of Spodoptera frugiperda (Sf9) cells with a baculovirus encoding the 5-HT1A receptor. Receptor expression was confirmed by immunoblot. Some of the receptors were functional, showing guanine nucleotide-sensitive binding to the specific agonist ligand [3H]8-hydroxy-2-(di-n-propylamino)-1,2,3,4-tetrahydronaphthalene. Peak expression (=150 fmol/mg of membrane protein) was attained at 72-96 h post-infection. 5-HT1A increased covalent binding of [32P]GTP-azidoanilide to a 40 kDa band, which was identified as a G protein by nucleotide blocking, Mg2+ dependence, and immunoblot and immunoprecipitation studies. The band comigrated with 1) pertussis toxin substrate(s), and 2) a band recognized by two Go, antisera and one common to heterotrimERIC G protein α-subunits, but not by sera specific for G,, or G,,. Labeled species could be precipitated with a G, antisera. 5-HT1A increased labeling of the band was prevented by preincubation with pertussis toxin. These studies suggest that the 5-HT1A receptor couples effectively to native insect cell G,,like proteins.

The baculovirus expression system has proved to be a convenient and powerful means of attaining high level expression of many signal transduction components, including nuclear (1) and cell surface receptors (2-7) and G proteins (9-11). One especially important application of this method is for overexpression and purification of plasma membrane receptors for biochemical and reconstitution studies (2-8). In designing such studies, one must take into account the possibility that the heterologously expressed receptors may couple to endogenous G proteins. This is particularly important if the purified receptors are to be used for functional reconstitution studies with G proteins. Two groups recently showed that β-adrenergic receptor coupling to and activating an endogenous insect cell G protein analogous to mammalian G, in Sf91 cells, which in turn leads to an accumulation of cellular cAMP (2,6). Another group showed that the substance P receptor, which couples to G proteins from the G,, class of G proteins, can couple to an undefined G protein population in Sf9 cells (7). However, coupling of mammalian receptors to pertussis toxin-sensitive G proteins in insect cells has been much more confusing. The gene for a putative insect homologue to mammalian G, has been cloned from a Drosophila melanogaster library (12,13) and a potential insect G, has been reported in Sf9 cells (2,8), so there is a possibility that mammalian receptors could also couple to this class of G protein in insect host cells. Evidence for an intact signaling pathway including mammalian receptor, inhibitory (pertussis toxin-sensitive) G protein, and effector is scant (14,15). Oker-Bloom et al. have reported that the α7-C4 adrenergic receptor inhibits forskolin-stimulated cAMP accumulation in Sf9 cells, although no attempt was made to characterize the responsible G protein(s). The very presence of pertussis toxin-sensitive G proteins in insect cells has been controversial. Quehenberger et al. (5) recently showed that chemoattractant Met-Leu-Phe receptors expressed in high density in insect cells did not activate GTPase activity, nor could they be demonstrated to possess GTP-sensitive high affinity agonist binding sites. They were also unable to show any pertussis toxin substrates in membranes derived from insect cells using an ADP-ribosylation assay. Those findings were proposed as evidence that the Sf9 cell lacks a major category of G proteins (G,). In contrast, Richardson and Hosey (8) recently demonstrated that hm,, muscarinic receptors activated GTPase activity and increased GTPyS binding to membranes derived from Sf9 cells. These linkages were completely sensitive to small doses of pertussis toxin (7). However, distal signaling events were not reported. Vasudevan et al. (15) showed that the rat m,, muscarinic receptor activated potassium currents when expressed in insect cells, and that this activation was abolished by preincubation with extremely high doses of pertussis toxin (2 μg/ml for up to 72 h). Finally, two groups have previously suggested in footnotes that G,,-like proteins were present in their Sf9 cell preparations (2,8), but supporting data were not presented in those manuscripts.

Thus, the presence within Sf9 cells of pertussis toxin-sensitive G, proteins has been controversial. The current studies...
provide evidence that the 5-HT<sub>1A</sub> receptor, which couples particularly to pertussis toxin-sensitive G proteins in mammalian cells (16, 17), can couple to an endogenous G<sub>i</sub> linked receptor in S<sub>F9</sub> cells. These observations should be considered for any G<sub>i</sub>-linked receptor that is expressed in insect cells for the purpose of purification and reconstitution with G proteins.

**EXPERIMENTAL PROCEDURES**

**Materials**—Most materials and reagents were obtained from Sigma (St. Louis, MO), with the exception of polyethylene glycol 8000. Other materials were obtained from sources listed below.

**Characteristics of Antisera Used for This Study—**Antisera used for these studies were as follows. Antiserum 982 was raised against the carboxyl terminal peptide K<sup>194</sup>ENLKDGCGL<sup>208</sup> of G<sub>i2</sub>, and G<sub>ai</sub>, both of which recognize G<sub>i2</sub>. Antiserum 978 recognizes an internal sequence of G<sub>i2</sub> (L<sup>149</sup>DRIAAQPNY<sup>154</sup>), for which it is highly specific. Antiserum 977 was raised against the carboxyl-terminal segment of K<sup>194</sup>NLKDECGY<sup>206</sup> and interacts specifically with G<sub>i2</sub>. Antiserum 975 was raised against the same sequence, but recognizes both G<sub>i2</sub> and G<sub>ai</sub>. Antiserum 951 was raised against the carboxyl-terminal fragment of G<sub>i2</sub> (R<sup>200</sup>MHLRQYELL<sup>208</sup>), for which it is highly specific. These sera were raised in one of the authors' laboratories (T. W. G.). Specificity was evaluated by immunoblot and immunoprecipitation assays using purified or partially purified G proteins expressed in bacteria (21).

**Photoaffinity Labeling of Insect Cell G Proteins—**The Pansorbin was isolated by centrifugation at 12,000 g for 5 min. Then washed once by resuspension in immunoprecipitation buffer 1 followed by centrifugation. Each pellet was resuspended in 50 μl of 2 x sample buffer, freeze-thawed, heated to 60 °C for 5 min, cooled to room temperature, and subjected to SDS-PAGE and immunoblot.

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**Antigenic Conclusions—**Antisera used for these studies were as follows. Antiserum 982 was raised against the carboxyl terminal peptide K<sup>194</sup>ENLKDGCGL<sup>208</sup> of G<sub>i2</sub>, and G<sub>ai</sub>, both of which recognize G<sub>i2</sub>. Antiserum 978 recognizes an internal sequence of G<sub>i2</sub> (L<sup>149</sup>DRIAAQPNY<sup>154</sup>), for which it is highly specific. Antiserum 977 was raised against the carboxyl-terminal segment of K<sup>194</sup>NLKDECGY<sup>206</sup> and interacts specifically with G<sub>i2</sub>. Antiserum 975 was raised against the same sequence, but recognizes both G<sub>i2</sub> and G<sub>ai</sub>. Antiserum 951 was raised against the carboxyl-terminal fragment of G<sub>i2</sub> (R<sup>200</sup>MHLRQYELL<sup>208</sup>), for which it is highly specific. These sera were raised in one of the authors' laboratories (T. W. G.). Specificity was evaluated by immunoblot and immunoprecipitation assays using purified or partially purified G proteins expressed in bacteria (21).

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Expression of human 5-HT₁A receptors in insect cell membranes. Membranes were prepared by mechanical disruption of cells in lysis buffer as described under "Experimental Procedures." Panel A, membranes were then run under reducing conditions on 10% SDS-PAGE gels and subjected to either silver staining or immunoblot with affinity-purified anti-5-HT₁A receptor IgG. The left hand panel shows the results of a silver staining experiment. The standards (Stds) used were (from top: myosin, phosphorylase b, bovine serum albumin, ovalbumin, and carbonic anhydrase). The ovalbumin standard is prestained with yellow dye and runs typically at ~53 kDa when compared with non-dyed ovalbumin. Each lane contained 30 µg of control (C), 24, 48, and 72 h except for the 96 h lane, which contained 50 µg of protein. The right hand gel was treated identically, except that it was subjected to immunoblot with antiserum 5-HT₁A/5-HT₁B (23). Induction of a new protein of about 46 kDa (arrow) was noted on both the silver stain and immunoblot. Similar results were obtained in two other experiments. The theoretical core molecular mass of the 5-HT₁A receptor is 46 kDa (18). Corresponding agonist ligand binding experiments with [³H]8-OH-DPAT (Table 1; see legend of Fig. 1 for more details) also demonstrated the following amount of receptor at each time: control (none), 24 h (none detected), 48 h (45 ± 3 fmol/mg protein), 72 h (106 ± 15 fmol/mg protein), and 96 h (180 ± 23 fmol/mg protein). Panel B, comparison of relative amounts of ligand binding (left axis, white bars) and densitometrically quantified immunoreactive receptor bands (right axis, gray bars).

Fig. 1.

Results

Expression of human 5-HT₁A Receptors in Insect Cells—Expression of receptor protein was confirmed by immunoblot with a specific affinity-purified antipeptide IgG raised against a portion of the putative third intracellular loop of the human 5-HT₁A receptor (Fig. 1A). Immunoblottable protein was detectable in the membrane fraction at 48–96 h. In fact, a silver-stained band of similar mobility was also seen at 48–96 h only in cells infected with the construct bearing the 5-HT₁A receptor. The functionality of some of the expressed receptors was confirmed by ligand binding experiments. Surprisingly, relatively little ligand binding was detected (~100–150 fmol of receptor/mg of protein) compared with expression levels reported for several other G protein-coupled receptors (2–8), which ranged from 20 to nearly 250-fold higher than in the current studies. We expected much higher levels of binding based on the silver stain results (Fig. 1A). It seems likely that a substantial fraction of the membrane-bound receptors are unable to bind [³H]8-OH-DPAT, possibly due to improper or inefficient processing. We compared the relative amounts of the immunoreactive receptors by scanning densitometry with ligand binding results (Fig. 1B). The comparison suggests that a relatively constant fraction of the expressed immunoreactive receptors are capable of binding to [³H]8-OH-DPAT. Although it is likely that many (or most) of the expressed receptors are not functional, a level of 100–150 fmol of functional receptor/mg of protein falls well within the physiological range of expression of the 5-HT₁A receptor in brain (17) (see legend of Fig. 1 for more details). We next determined whether the human 5-HT₁A receptor coupled to G proteins in the insect host cells.

Presumptive Evidence for Coupling of 5-HT₁A Receptors to G Proteins: Guanine Nucleotide Sensitivity of Agonist Binding—The ability of guanine nucleotides to reduce the high affinity binding of the specific agonist ligand [³H]8-OH-DPAT to the 5-HT₁A receptor was determined in two different assays. As shown in Fig. 2A, 10 µM GTP shifted the Scatchard plot of the binding isotherm for [³H]8-OH-DPAT from a predominantly...
The majority of 5-HT_1 receptors capable of binding [3H]8-OH-DPAT increases in AA-GTP labeling in insect cell membranes (GTPyS coupled proteins in the insect cell membranes with a photoreactive GTP analogue). Their approach takes advantage of the observation that receptor activation can increase the GTP-binding rate of some G protein α-subunits. As shown in Fig. 3, 5-HT treatment of membranes incubated with AA-GTP caused a dose-dependent 2–3-fold increase in the labeling of a 40 kDa band, which presumably represents G protein(s) activated by the 5-HT_1 receptor. In 12 experiments, 5-HT also appeared to increase the labeling of a 65 kDa band, but this observation was not consistently reproduced. In fact, in the absence of GDP, the higher band was often not visualized. As previously observed for opioid receptors in NG108–15 membranes, GDP was required for agonist-promoted G protein labeling of the 40 kDa band to be observed (31). Those authors ascribed this phenomenon to the observations of Florio and Sternweis (38) suggesting that receptor-activated G proteins have a high affinity for GTP and its analogs, whereas non-activated G proteins have a higher affinity for GDP. Thus, this effect has been attributed to a selective suppression by GDP of basal AA-GTP binding to G proteins that are not coupled to receptors. For the 5-HT_1A receptor expressed in insect cells, the best results were obtained at a GDP concentration of 10 μM. The half-maximal dose for the stimulation of labeling determined by scanning densitometry and curve fitting was 80 ± 15 nm, indicating a potential physiologic interaction between the 5-HT_1A receptor and the G protein. In order to firmly establish that the labeling of the 40 kDa band was increased through a pathway involving the 5-HT_1A receptor, several experiments were performed on non-infected cells and cells infected with a baculovirus construct bearing the human β2-adrenergic receptor (provided by Dr. Bob Lefkowitz). There was no 5-HT-induced increase in labeling of the 40 kDa band in either case (n = 5 and 3, respectively, Fig. 3C). Furthermore, in cells infected with the 5-HT_1A receptor-bearing baculovirus construct, the labeling could be blocked by preincubation with the 5-HT_1A receptor antagonist spiperone at 100 μM (Fig. 3).

CHARACTERIZATION OF THE PUTATIVE 40-KDA G PROTEIN LABELED BY AA-GTP—In order to establish that the 40 kDa band is a G protein, we performed experiments to determine the sensitivity of the labeling to nucleotides and magnesium. Mammalian G proteins typically are sensitive both to guanine nucleotides and magnesium and are relatively insensitive to adenine nucleotides. Labeling of the 40 kDa band could be greatly attenuated by GDP, GTP, and GDP, but not by App[γS]GTP, GP, and GDP, and GTP, not by App[γS]GTP, GP, and GDP, and not shown), supporting the notion that the band is a G protein. We were also able to show a clear magnesium dependence (EC_50 ~ 5 mM) of the incorporation of AA-GTP into the 40 kDa band (n = 5, not shown). Those studies demonstrate that the labeling of the 40 kDa band conforms well with properties expected of mammalian G proteins. To further characterize the nature of the 40 kDa band, membranes were also subjected to immunoblot with a panel of antibodies directed against various regions of mammalian Gα and Gβγ α-subunits (Fig. 4). Those results showed no 40 kDa immunoreactivity with antibodies raised against the carboxyl-terminal regions of Gα1, Gα2, Gα12 or Gα13 (Fig. 4A). In contrast, antisera raised against the GTP-binding domain common to mammalian G protein α-subunits, and two distinct regions of Gα recognized a prominent 40 kDa band in insect cell membranes (Fig. 4B). Because that band comigrates with that labeled by AA-GTP, it suggested, but did not prove, that they may be the same protein. Fig. 4C demonstrates that an antisera against the GTP-binding region of Gα (P280) is capable of immunoprecipitating the 40 kDa band, as is an antiserum (976) that reacts against Gα/GTP. Non-immune serum does not immunoprecipitate the 40 kDa band. Those findings demonstrate a clear physical association between the G protein labeled by AA-GTP and the Gα immunoassay reactive species. They confirm that the 40 kDa band, the AA-GTP labeling of which was enhanced by the 5-HT_1A receptor, is a G protein with antigenic...
sites that conform to domains of mammalian Gαi.

If the labeled band is truly analogous to mammalian Gαi, it should be a substrate for pertussis toxin-catalyzed ADP-ribosylation. As shown in Fig. 5A, there is a prominent 40-kDa band in S9 cell membranes ADP-ribosylated in the presence, but not in the absence, of pertussis toxin. Immunoblot analysis of CHO-K1 cell membranes in our laboratories (not shown), and in two others (39, 40), have demonstrated that the major pertussis toxin substrates in CHO cells are Gαi2 and Gαi3, although another group has suggested that the major substrates in CHO-DG44 cells are Gαi2 and Gαi1 (41). Regardless, the S9 cell substrate migrates with a slightly smaller mass than substrates labeled in CHO-K1 cell membranes, a slightly larger mass than bovine brain Gαi3, and appears to be present in about half the amount in S9 cells compared with CHO cells. Pertussis toxin-facilitated ADP-ribosylation of the 40-kDa substrate from S9 cell membranes strongly suggests that the substrate is a Gαi-like protein, but does not prove it. Therefore, we next used antisera P960 and 976 to immunoprecipitate the pertussis toxin-labeled band. As shown in Fig. 5B, these antisera but not non-immune serum precipitated the band, strongly supporting the notion that it is a Gαi-like protein. Because neither serum P960, nor serum 976 completely cleared the AA-GTP-labeled band from the soluble preparations (each cleared 30–50%), we performed serial immunoprecipitations with 976 from the remaining supernatants to determine if the ADP-ribosylated bands could be completely cleared. As shown in Fig. 5C, a second immunoprecipitation cleared a further 50% of the labeled band, whereas a third did not further clear the band. The inability to completely clear the band could be due to a relative inefficiency of the serum, or to the presence of another 40-kDa G protein pool. Our data do not allow us to distinguish between those possibilities. However, our results confirm that at least 75% of the AA-GTP-labeled 40-kDa substrates are Gαi-like proteins.

In order to firmly establish a link between the AA-GTP-labeled 40 kDa band and that labeled by pertussis toxin in S9 cells, we pretreated intact cells with pertussis toxin in an attempt to eliminate the 5-HT1A receptor-augmented component of AA-GTP labeling. Unfortunately, we were not able to reduce the amount of pertussis toxin substrate by more than 25–50% as assessed by subsequent membrane ADP-ribosylation studies (not shown). Cells were treated with up to 2 μg/ml of toxin for up to 48 h. Because of two previous reports of pertussis toxin-sensitive signaling pathways in S9 cells (8, 15), we tested six different batches of pertussis toxin from two distributors (Sigma and List) and two different batches of CHO cells (one from Invitrogen and one from Bob Lefkowitz) with similar results. We performed experiments in serum-supplemented media (n = 3) and unsupplemented defined media (n = 4) with similar results. Those same batches of pertussis toxin were able to effectively catalyze the membrane reaction after preactivation (Figs. 5 and 6), and the toxin itself appeared to possess a similar enzymatic capability. To evaluate the unlikely possibility that all six batches of toxin were accidentally preactivated by denaturation or exposure to DTT (thus, causing separation of the enzymatically active A component from the B component important for cellular uptake), we performed control experiments on intact CHO cells. Those studies showed that treatment of intact CHO cells with only 100 ng of the toxin for 4 h completely eliminated subsequent labeling of membrane pertussis toxin substrates (n = 3, not shown). Our conclusion was that under our experimental conditions, pertussis toxin was either poorly taken up into S9 cells or was poorly activated by them.

In order to answer the question of the pertussis toxin sensitivity of the AA-GTP labeling, we needed to circumvent the technical problem described above. We did so by treating membranes derived from S9 cells infected with the 5-HT1A receptor construct with preactivated pertussis toxin, then subjecting
**FIG. 4. Immunoblots with a panel of antisera raised against specific mammalian G protein α-subunits and immunoprecipitation of the AA-GTP-labeled band with G protein antisera.** Panel A, aliquots of membranes prepared from Sf9 cells and rat adipocytes were subjected to immunoblot and visualization with 12959 of the AA-GTP-labeled band with G protein antisera. Panel B, for these experiments, purified G protein standards derived from bovine brain (from Dr. Pat Casey) were used as controls. Panel C, aliquots of membranes prepared from Sf9 cells were subjected to AA-GTP labeling, solubilization, and immunoprecipitation as described under "Experimental Procedures." The amount of membrane protein analyzed under each condition is noted in the figure. For the sera in this panel, IgG fractions were isolated from serum by protein A high performance liquid chromatography prior to immunoblot and resuspended in a volume of phosphate-buffered saline equal to the amount of serum from which they were derived. Dilutions of the IgG fractions used for these studies were 1:4000 for 978 and 982, 1:20,000 for 951, and 1:40,000 for 977. Autoradiograms were exposed to Kodak X-AR film for less than 24 h at -80 °C. Panel B, for these experiments, purified G protein standards derived from bovine brain (from Dr. Pat Casey) were used as controls. Dilutions of crude sera used for these blots were 1:2000 for P960, 1:400 for U46, and 1:16,000 for 976. Autoradiograms were exposed to Kodak X-AR film for 5 h at -80 °C. Blots were repeated at least three times for each serum or IgG fraction. Panel C, aliquots of membranes prepared from Sf9 cells (40 μg) were subjected AA-GTP labeling, solubilization in 100-μl volumes, and immunoprecipitation as described under "Experimental Procedures." P960 was used at a 1:40 dilution and 976 and non-immune serum at 1:100 dilution. After preclearing with Pansorbin, the supernatants were subjected to SDS-PAGE or reacted with antisera. The supernatants represent the starting material from which the immunoprecipitates were derived. Autoradiograms are representative of three to five experiments and were exposed to Kodak X-AR film overnight at -80 °C.

In order to eliminate any effects of DTT (with which the pertussis toxin was preactivated) on the disulfide bonds within the receptor, the preactivated pertussis toxin was dialyzed against 30 mM HEPES, pH 7.5, 100 mM NaCl, 100 mM EDTA, 1 mM benzamidine, 50 μM leupeptin using a Centricon 3000 MW cut-off filter before addition to the membrane preparation. The results of those experiments (Fig. 6) showed that pretreatment with pertussis toxin eliminated the ability of the 5-HT1a receptor to increase the AA-GTP-labeling of the 40-kDa substrate. Those findings provide further evidence that the 5-HT1a receptor in Sf9 cells couples to a pertussis toxin-sensitive G-like protein and increases its labeling by AA-GTP. It does not rule out coupling to other G proteins in those cells.

Final proof of the coupling of the 5-HT1a receptor to the G-like protein requires a clear demonstration of physical concordance between the immunoreactive G-like protein and the 40 kDa band, the labeling of which is increased by the 5-HT1a receptor. That proof is provided in Fig. 7, in which membranes were exposed to AA-GTP in the presence or absence of 1 μM 5-HT, solubilized and then immunoprecipitated with serum 976. After immunoprecipitation, the increase in labeling by 5-HT is readily apparent. Thus, we have shown physical concordance between a 40 kDa band that is labeled by AA-GTP and ADP-ribosylated by pertussis toxin, which has immunoreactivity in immunoblot and immunoprecipitation assays with Gα antisera, and which shows increased labeling by GTP when the 5-HT1a receptor is activated. The increased labeling is present only when Sf9 cells are infected with 5-HT1a receptor (but not β2 receptors) and are treated with 5-HT and can be blocked by the 5-HT1a receptor antagonist spiperone.

**DISCUSSION**

The baculovirus/insect cell system has become a powerful tool for the expression of various signal transduction components, including G protein-coupled receptors. It has already been demonstrated that β-adrenergic receptors can stimulate cAMP accumulation through an insect cell G protein analogous to mammalian Gα (2, 6). Functionally important events such as agonist-induced phosphorylation of hM1 muscarinic receptors (8) and various processing events of β2-adrenergic receptors have also been demonstrated to occur in insect cells (2, 3, 8, 34). However, the nature of the Sf9 cell G proteins that couple to receptors normally coupled to mammalian Gα is unclear. The
current studies demonstrate a functional coupling of human 5-HT₁₆ receptors to a pertussis toxin-sensitive G protein pool in Sf9 insect cells, thus demonstrating that this class of receptors can modulate G protein function in these host cells. The coupling of the 5-HT₁₆ receptors to pertussis toxin-sensitive G proteins in insect cells will provide a unique opportunity to examine the relationship between G protein structure and function among the G proteins and their "cognate" receptors such as the 5-HT₁₆ receptor. It will be informative to compare the sequences of mammalian Gₛₙ₆ proteins and the endogenous insect cell G proteins that couple to the 5-HT₁₆ receptor.

These studies underscore the amazing conservation of structure and function among the G proteins and their "cognate" receptors throughout the animal kingdom. This conservation was previously demonstrated both by the ability of β-adrenergic receptors to stimulate cAMP accumulation through an insect cell G protein analogous to mammalian Gₛₙ₆ (2, 6), and by the ability of Drosophila 5-HT₁₆ receptors expressed in mammalian NIH-3T3 cells to inhibit murine adenyl cyclase activation through a pertussis toxin-sensitive G protein pool (35, 36).

5-HT₁₆ receptors have already been shown to be somewhat "promiscuous" in coupling to mammalian Gₛₙ₆ proteins (16, 21, 37). The current studies show that human 5-HT₁₆ receptors can couple functionally to endogenous G proteins expressed in insect cells. These findings are particularly important because

FIG. 5. Pertussis toxin-mediated ADP-riboylation of a 40-kDa substrate in Sf9 cells. Experiments were performed as described under "Experimental Procedures." Panel A, aliquots of membranes prepared from CHO cells and Sf9 cells were subjected to ADP-riboylation in the absence or presence of pertussis toxin. Aliquots were loaded onto 12% SDS-PAGE gels in duplicate amounts as noted in the figure. After electrophoresis, gels were washed extensively in 20% methanol, 10% acetic acid, dried, and exposed to Kodak X-AR film at room temperature (n = 5). The gel depicted was exposed for 2 h. Panel B, aliquots of membranes prepared from Sf9 cells (25 μg) were subjected to AA-GTP-labeling, solubilization in 100-μl volumes, and immunoprecipitation as described under "Experimental Procedures." Aliquots were pre cleared with protein A-Sepharose, then were incubated with non-immune serum, 976 or P960 at 1:100 dilutions overnight at 4 °C. Another 20-μl aliquot of 50% (w/v) protein A-Sepharose beads in phosphate-buffered saline was added to each sample and incubated on a rotator for 30 min at room temperature. Immune complex precipitates were separated from the supernatants by centrifugation (500 x g for 1 min). The immunoprecipitates were rapidly washed thrice with ice-cold phosphate-buffered saline followed by centrifugation. The supernatants represent what remains from the starting material from which the immunoprecipitates were derived. Autoradiograms are representative of three experiments and were exposed to Kodak X-AR film for 2 days at −80 °C. Panel C, serial immunoprecipitations of ADP-riboylated proteins were performed with serum 976. After IP 1, supernatant and precipitates were subjected to SDS-PAGE. The left-hand two lanes show that equal amounts of ADP-riboylated proteins were in the supernatant and immunoprecipitate. An aliquot corresponding to the remaining supernatant from IP 1 was added to another aliquot of 976 (1:100) and subjected to immunoprecipitation (IP 2), again showing clearing of about 50% of the labeled band (middle two lanes). A third precipitation (IP 3) from an aliquot corresponding to the supernatant remaining after IP 2 was subjected to further treatment with 976. IP 3 did not precipitate any further ADP-riboylated proteins (right two lanes).

FIG. 6. Effect of pertussis toxin-facilitated ADP-riboylation of Sf9 cell membranes on the ability of the 5-HT₁₆ receptor to increase AA-GTP labeling. Membranes from Sf9 cells (50 μg) were preincubated with vehicle (lanes A-D and I) or pertussis toxin (2 μg) for 1 h at room temperature as described in the protocol for ADP-riboylation, except that no [³²P]NAD⁺ was included in the incubation mix. Those membranes were then either subjected to ADP-riboylation in the presence of [³²P]NAD⁺ and pertussis toxin (2 μg) for 1 h at room temperature (lanes I and J) or to photolabeling with AA-GTP in the absence (lanes A, B, E, and F) or presence (lanes C, D, G, and H) of 1 μM 5-HT. Samples were then subjected to 12% SDS-PAGE and autoradiography for 24 h at −80 °C. The entire 50-μg sample was loaded for all photolabeling conditions (lanes A-H), but only 10 μg for each ADP-riboylation (lanes I and J). The autoradiogram is representative of three experiments with nearly identical results.
the baculovirus/insect cell expression system has become a popular method of expressing G protein-coupled receptors for purification and reconstitution with mammalian G proteins. The coupling of 5-HT\textsubscript{1\textalpha} receptors to G proteins in insect cells is exquisitely sensitive to guanine nucleotides. In the current studies, \textsim70% of receptor binding to 1 \textmu M [3H]-8-OH-DPAT was guanine nucleotide-sensitive. Moreover, 5-HT-induced photoaffinity labeling of 40-kDa putative G\textsubscript{s} protein(s) was demonstrated in membranes derived from insect cells infected with baculovirus bearing the DNA of the 5-HT\textsubscript{1\textalpha} receptor. The 40 kDa band(s) was shown by a variety of techniques to possess biochemical features of G proteins, G\textsubscript{s}-like immunoreactivity, and pertussis toxin sensitivity in membranes.

It was also perplexing to note that the receptor-modified G protein(s) appeared to be insensitive to pertussis toxin treatment of intact cells, yet were susceptible to pertussis toxin treatment of membranes. Our experiments seem to support the possibility that insect cells either are poorly able to internalize or activate the pertussis toxin complex under our experimental conditions. The Sf9 cells used were commercially obtained from a common supplier and also from a colleague, and it is not easy to directly compare our results with those of Richardson and Hosey, who previously showed that low doses of pertussis toxin abolished coupling of the hm, muscarinic cholinergic receptor to G proteins in insect cells (8). We used six different batches of pertussis toxin at very high doses with prolonged incubations without a discernible effect on intact Sf9 cells. Those same batches were used to treat intact CHO cells bearing the 5-HT\textsubscript{1\textalpha} receptor. Preincubation with 100 ng/ml for 4 h completely eliminated pertussis toxin substrates and blocked the ability of the 5-HT\textsubscript{1\textalpha} receptor to inhibit adenylylcyclase in CHO cells (not shown). Therefore, it is highly unlikely that our multiple batches of toxin were defective. Another possibility is that the 5-HT\textsubscript{1\textalpha} receptor is tightly precoupled to the G proteins in insect cells, thus blocking access of the toxin to the carboxyl terminus of the G proteins. Although we did not directly test that hypothesis, we think it unlikely because of the prolonged incubations of intact cells with pertussis toxin used in our experiments, and because such would imply a stoichiometric excess of functional receptor over G protein. On the one hand, such an excess of functional receptors over G proteins would seem to be unlikely in the face of the abundant 40-kDa G protein present in Sf9 cells and the low level of receptors capable of binding ligand in these studies. On the other hand, because a large amount of receptors incapable of binding ligand appear to be expressed in the membranes of Sf9 cells (Fig. 1), there remains the remote possibility that agonist-independent tight precoupling of those 5-HT\textsubscript{1\textalpha} receptors to G proteins could indeed block access of pertussis toxin to the carboxyl terminus cysteine acceptor site of the G\textsubscript{s}-like proteins in Sf9 cells.

The current studies clearly document the presence of G\textsubscript{s}-like proteins in Sf9 insect cells based on pertussis toxin labeling, nucleotide and Mg\textsuperscript{2+}-sensitive AA-GTP labeling, immunoblot analyses, and immunoprecipitation of both pertussis toxin- and AA-GTP-labeled 40-kDa substrates. On the surface, these results directly conflict with those of Quehenberger \textit{et al.} (5), who showed a lack pertussis toxin substrates and lack of coupling of chemotactic G\textsubscript{M}-Leu-Phe receptor to endogenous G proteins in Sf9 cells. There is no obvious explanation for the discrepancies between their findings and ours, although we did find that inclusion of lubrol in the ADP-ribosylation assays dramatically increased the pertussis-toxin facilitated labeling of the 40 kDa band. It should also be noted that two groups have previously suggested in footnotes that G\textsubscript{s}-like proteins were present in their Sf9 cell preparations (2, 8), but supporting data was not presented in those manuscripts.

Our studies point out the need for special care to eliminate the effects of endogenous G proteins when designing experiments in which such coupling might complicate interpretation. The fact that G protein coupling was not demonstrated for G\textsubscript{M}-Leu-Phe receptors expressed at very high density in Sf9 cells (more than 200-fold greater than in the current experiments) illustrates that a careful examination is warranted for each new receptor heterologously expressed in insect cells (5). Moreover, an effect should not be deemed insensitive to pertussis toxin unless care is taken to document that all pertussis toxin substrate has been inactivated as assessed by membrane ADP-ribosylation studies.

The current studies also underscore the high degree of conservation of structure and function among G proteins and their "cognate" receptors throughout the animal kingdom, and further validate the insect cell expression system as a relevant physiological model. They also present the first direct evidence that the 5-HT\textsubscript{1\textalpha} receptor can couple to an endogenous insect cell G\textsubscript{s}-like protein.

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Addendum—Ng (Ng, G. Y. K., George, S. R., Zastawny, L. R., Caron, M., Bouvier, M., Dennis, M., and O'Dowd, B. F. (1993) \textit{Biochemistry} \textbf{32}, 11727–11733) recently reported the presence of a 41-kDa pertussis toxin substrate and G\textsubscript{s}-type agonist binding to 5-HT\textsubscript{1\textalpha} receptors in Sf9 cells.

REFERENCES

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Sf9 Cell G\textsubscript{x}-like Protein