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Effects of Sulfhydryl-Modifying Reagents, 3-Nitro-2-pyridinesulfenyl Compounds, on the Coupling between Inhibitory Receptors and GTP-Binding Proteins G_i/G_o in Rat Brain Membranes

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SUMMARY

To gain insight into the coupling mechanism of inhibitory receptors, 5-hydroxytryptamine$_{1A}$ receptors and α_2-adrenoceptors, with GTP-binding proteins (G proteins) in the central nervous system, we examined the effects of two 3-nitro-2-pyridinesulfenyl compounds, *S*-(3-nitro-2-pyridinesulfenyl)-L-cysteine [Cys(Npys)] and *N*-*t*-butoxy-carbonyl-*S*-(3-nitro-2-pyridinesulfenyl)-L-cysteine [Boc-Cys(Npys)], on 1) specific binding of [^{3}H]8-hydroxy-2-(di-*n*-propylamino)tetralin (8-OH-DPAT) (5-hydroxytryptamine$_{1A}$ agonist) and [^{3}H]clonidine (α_2-agonist) to rat brain membranes, 2) [^{35}S]guanosine 5′-*O*-(3-thio)triphosphate (GTPγS) binding, and 3) pertussis toxin (islet-activating protein) (IAP)-catalyzed ADP-ribosylation of purified G_o (an IAP-sensitive G protein present in abundance in the mammalian brain). Treatment with Cys(Npys) led to decreased [^{3}H]8-OH-DPAT and [^{3}H]clonidine binding, similar to the inhibitory effects of IAP and *N*-ethylmaleimide (NEM) on such binding. However, further treatment of Cys(Npys)-pretreated membranes with dithiothreitol completely abolished the inhibitory effect of Cys(Npys) on the binding of both ligands. On the other hand, treatment with Boc-Cys(Npys) inhibited the effect of several GTP analogs (GTPγS, guanylyl-imidodiphosphate, guanylyl-(β,γ-methylene)-diphosphate, and GTP) on [^{3}H]8-OH-DPAT and [^{3}H]clonidine binding. Dithiothreitol and mercaptoethanol treatment of Boc-Cys(Npys)-pretreated membranes did not lead to a recovery of the effect of GTP analogs on agonist binding. Regardless of the presence or absence of GTPγS, agonist binding to Boc-Cys(Npys)-pretreated membranes was decreased by further addition of NEM or Cys(Npys). Cys(Npys) blocked [^{35}S]GTPγS binding as well as IAP-catalyzed ADP-ribosylation in purified G_o. In contrast, Boc-Cys(Npys) partially inhibited ADP-ribosylation and did not affect [^{35}S]GTPγS binding. These results suggested that Cys(Npys) modifies the receptor-coupling domain in G proteins, followed by the uncoupling of inhibitory receptors from G proteins, similar to the effects of NEM and IAP. Boc-Cys(Npys), however, seems to stabilize the coupling state between the receptors and G proteins, thus abolishing the GTPγS effect.

In several types of receptors that couple with G proteins, the addition of guanine nucleotides causes the uncoupling of receptors from G proteins and a decrease in agonist binding. IAP catalyzes an ADP-ribosylation of the inhibitory G protein (G_i; $\alpha_{41}\beta\gamma$) and induces uncoupling of G_i from inhibitory receptors, e.g., α_2-adrenoceptors (1, 2), γ-aminobutyric acid$_B$ receptors (3), and 5-HT$_{1A}$ receptors (4), and attenuation of receptor-mediated inhibition of adenylate cyclase activity (5). Other G proteins that can be ADP-ribosylated by IAP have also been identified in the mammalian brain, e.g., G_o ($\alpha_{39}\beta\gamma$) and $\alpha_{40}\beta\gamma$ (6, 7). In addition, the presence of IAP-sensitive G proteins in the mammalian brain has been reported in the following order of predominance: $\alpha_{39}\beta\gamma \gg \alpha_{41}\beta\gamma > \alpha_{40}\beta\gamma$ (8). NEM, an irreversible sulfhydryl-alkylating reagent, suppresses agonist binding to inhibitory receptors and attenuates the agonist-stimulated inhibition of adenylate cyclase (9–11), similar to the effects of IAP, which are through the modification of cysteine at C-terminals of G_i/G_o.

Two Npys compounds, Cys(Npys) and Boc-Cys(Npys), are

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ABBREVIATIONS: G protein, GTP-binding protein; 5-HT, 5-hydroxytryptamine; Npys, 3-nitro-2-pyridinesulfenyl; Cys(Npys), *S*-(3-nitro-2-pyridinesulfenyl)-L-cysteine; Boc-Cys(Nyps), *N*-*t*butoxycarbonyl-*S*-(3-nitro-2-pyridinesulfenyl)-L-cysteine; 8-OH-DPAT, 8-hydroxy-2-(di-*n*-propylamino)tetralin; GTPγS, guanosine 5′-*O*-(3-thio)-triphosphate; Gpp(NH)p, guanylyl-imidodiphosphate; Gpp(CH)p, guanylyl-(β,γ-methylene)-diphosphate; IAP, islet-activating protein (pertussis toxin); G_o, GTP-binding protein present in abundance in the mammalian brain; DTT, dithiothreitol; NEM, *N*-ethylmaleimide; G_i and G_s, the inhibitory and stimulatory GTP-binding proteins of the adenylate cyclase system, respectively; DSP, dithiobis(succinimidyl propionate); PGE_2, prostaglandin E_2; BSA, bovine serum albumin; PMSF, phenylmethylsulfonyl fluoride; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel elctrophoresis; PBS, phosphate-buffered saline.

reversible sulfhydryl-modifying reagents (12, 13). It has been reported that Boc-Cys(Npys) completely eliminates papain enzymatic activity, which can be fully restored by addition of a potent reducing agent, tri-*n*-butylphosphine (10). In contrast, treatment with a cleavable bifunctional cross-linker, DSP, induces cross-linking of PGE_2 receptors and IAP-insensitive G proteins (14).

In the present study, we found that treatment with Cys(Npys) induces an uncoupling of inhibitory receptors, e.g., 5-HT_{1A} receptors and α_2-adrenoceptors, from G proteins, similar to the effects of treatment with NEM and IAP, but that treatment with Boc-Cys(Npys) results in stabilization of the coupling state between inhibitory receptors and G proteins.

Experimental Procedures

Materials. [*benzene ring*-^{3}H]Clonidine hydrochloride (1.54 TBq/mmol), [*propyl*-2,3-*ring*-1,2,3-^{3}H]8-OH-DPAT (5.79 TBq/mmol), [^{35}S]GTPγS (40.7 TBq/mmol), and [*adenylate*-^{32}P]NAD di(triethylammonium) salt (29.6 TBq/mmol) were purchased from New England Nuclear. NEM and DTT (Sigma), GTPγS, Gpp(NH)p, Gpp(CH)p, and GTP (Boehringer Mannheim), DSP and 2-mercaptoethanol (Nakarai Chemicals), affinity-purified horseradish peroxidase-labeled goat anti-mouse IgG and 4-chloro-1-naphthol (Bio-Rad), and Durapore GVHP sheets (Millipore) were purchased. IAP and Npys compounds were generously supplied by Kaken Pharmaceutical (Japan) and Sankyo (Japan), respectively.

Preparation of rat brain membranes and purification of G_o. Male adult Donryu rats were used. Fresh brains minus cerebella were homogenized with 10 volumes of 0.32 M sucrose, using a glass-Teflon homogenizer. The homogenate was centrifuged at 1,000 × *g* for 10 min and the supernatant was further centrifuged at 48,000 × *g* for 20 min. The pellet thus obtained was incubated at 30° for 15 min in 50 mM Tris·HCl buffer (pH 7.4) and then washed three times with the same buffer. The sedimented membranes were suspended (5–10 mg of protein/ml) in 50 mM Tris·HCl buffer (pH 7.4) and stored at −80°. G_o ($\alpha_{o3}\beta\gamma$) was purified from about 30 rat brains by the method of Katada *et al.* (6, 7). Protein was determined by the method of Lowry *et al.* (15), with BSA as the standard.

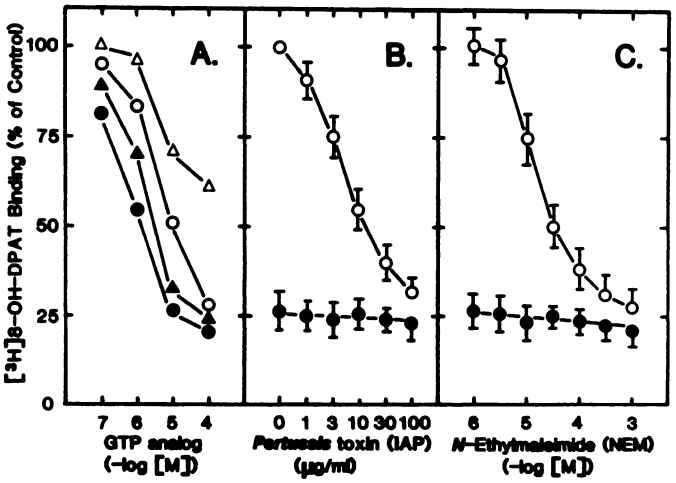

Fig. 1. Effects of GTP analogs, IAP, and NEM on [^{3}H]8-OH-DPAT binding to rat brain membranes. A, Brain membranes were incubated with 0.2 nM [^{3}H]8-OH-DPAT at 30° for 40 min in the presence of increasing concentrations of GTP analogs, GTP (○), GTPγS (●), Gpp(NH)p (▲), and Gpp(CH)p (△). Values are means of three determinations, which varied by <10%. Pretreatment with IAP (B) and NEM (C) was carried out as described in Experimental Procedures. The treated membranes were incubated with 0.2 nM [^{3}H]8-OH-DPAT in the presence (●) or absence (○) of 10 μM GTPγS. Values are means ± standard errors of three determinations.

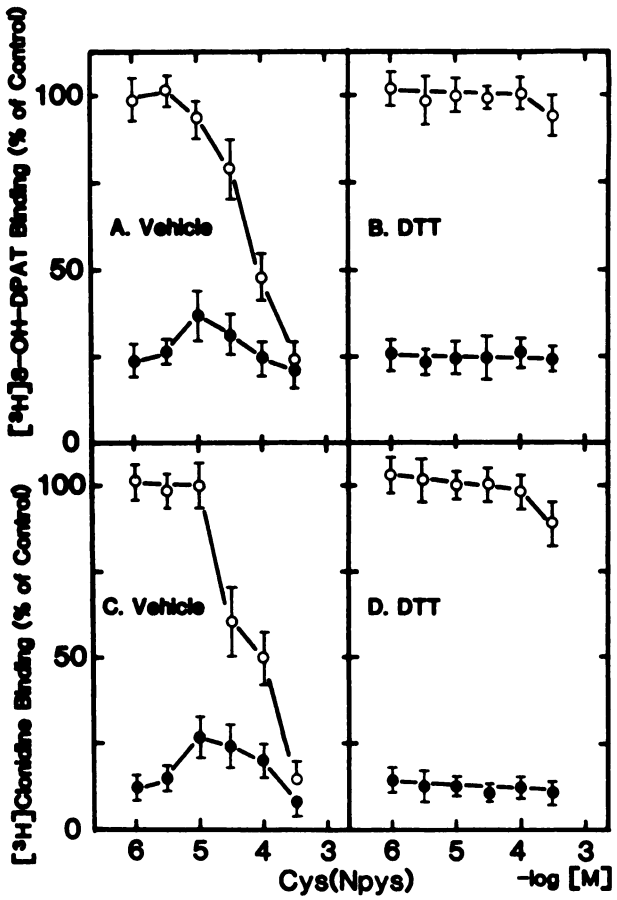

Fig. 2. Effect of Cys(Npys) pretreatment on [^{3}H]8-OH-DPAT and [^{3}H]clonidine binding. The rat brain membranes were incubated with the indicated concentrations of Cys(Npys) at 0° for 30 min, and then the membranes were washed. Cys(Npys)-pretreated membranes were further incubated in the presence (B and D) or absence (A and C) of 5 mM DTT, as described in Experimental Procedures. The treated membranes were incubated with 0.2 nM [^{3}H]8-OH-DPAT (A and B) or 0.5 nM [^{3}H]clonidine (C and D) in the presence (●) or absence (○) of 10 μM GTPγS at 30° for 40 min. Values are means ± standard errors of three determinations.

Treatment of brain membranes or purified G_o with NEM, Cys(Npys), Boc-Cys(Npys), or DSP. The rat brain membranes (about 2 mg of protein/ml), suspended in 50 mM Tris·HCl (pH 7.4), were incubated with various concentrations of NEM, Cys(Npys), Boc-Cys(Npys), or DSP at 0° for 30 min. The membranes were washed three times with 50 mM Tris·HCl buffer (pH 7.4). Pretreated membranes underwent a second incubation with or without 5 mM DTT or mercaptoethanol at 0° for 30 min and were then washed three times with 50 mM Tris·HCl (pH 7.4). The sedimented membranes were suspended in 50 mM Tris·HCl (pH 7.4).

Purified G_o contained 1 mM DTT, which was removed by gel filtration using a Sephadex G-25 column (1 × 25 cm) equilibrated with 20 mM Tris·HCl (pH 8.0) containing 0.7% CHAPS and 1 mM EDTA. The void fraction was used in this experiment. G_o obtained thusly was incubated with various concentrations of NEM, Cys(Npys), Boc-Cys(Npys), or DSP at 0° for 30 min. Pretreated G_o was further incubated with or without 5 mM DTT at 0° for 30 min. After these treatments, mixtures were assessed for [^{35}S]GTPγS binding and IAP-catalyzed [^{32}P]ADP-ribosylation activities.

5-HT_{1A} and α_2 agonist binding assay. The reaction mixture had a total volume of 200 μl and contained 50 mM Tris·HCl (pH 7.4), treated membranes (100–200 μg of protein), and each radiolabeled ligand, [^{3}H]8-OH-DPAT (0.2 nM) or [^{3}H]clonidine (0.5 nM), in the presence or absence of GTPγS, Gpp(NH)p, Gpp(CH)p, or GTP. After

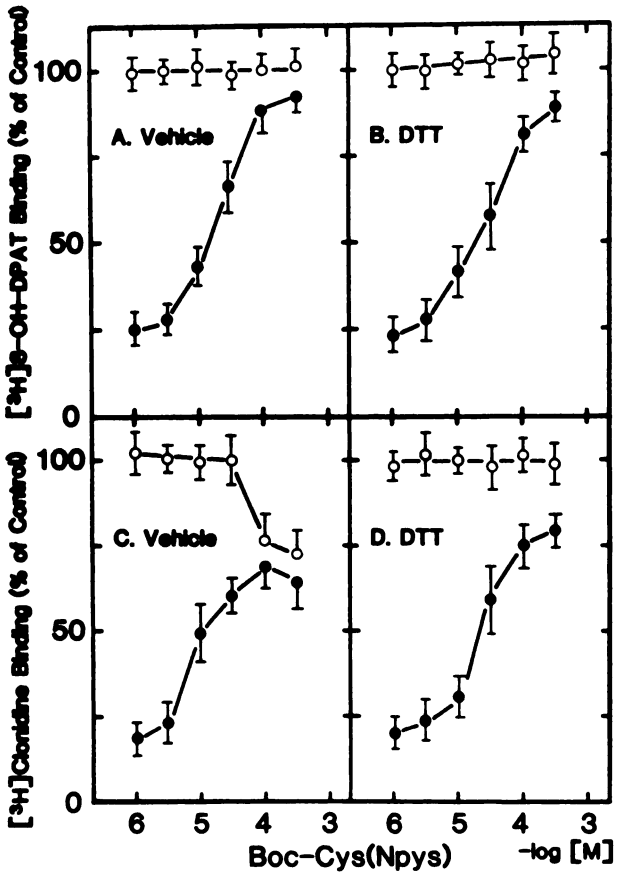

Fig. 3. Effect of Boc-Cys(Npys) pretreatment on [³H]8-OH-DPAT and [³H]clonidine binding. The rat brain membranes were incubated with the indicated concentrations of Boc-Cys(Npys) at 0° for 30 min, and then the membranes were washed. The pretreated membranes were further incubated in the presence (B and D) or absence (A and C) of 5 mM DTT, as described in Experimental Procedures. The treated membranes were incubated with 0.2 nM [³H]8-OH-DPAT and (A and B) or 0.5 nM [³H] clonidine (C and D) in the presence (●) or absence (○) or 10 μM GTPγS at 30° for 40 min. Values are means ± standard errors of three determinations.

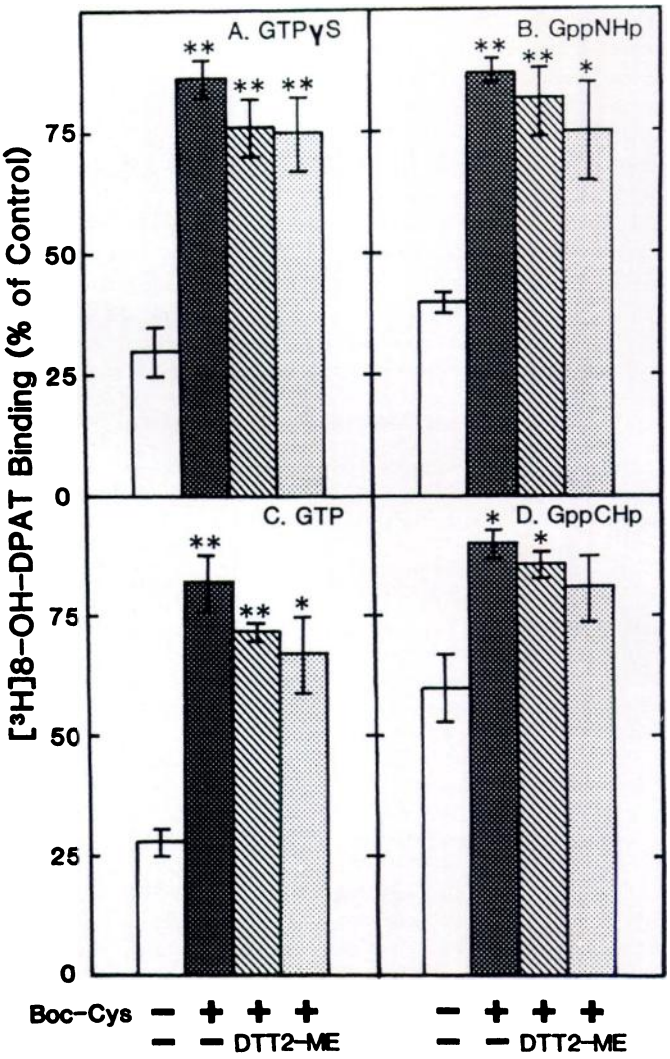

Fig. 4. Effects of GTP analogs and secondary treatment with reducing agents, DTT and 2-mercaptoethanol (*2-ME*), on [³H]8-OH-DPAT binding in Boc-Cys(Npys)-pretreated membranes. Boc-Cys(Npys)-pretreated membranes were further incubated without (■) or with 5 mM DTT (▨) or 5 mM 2-mercaptoethanol (□). After these treatments, control membranes (□) and treated membranes were incubated with 0.2 nM [³H]8-OH-DPAT in the presence of 10 μM GTPγS (A), 10 μM Gpp(NH)p (B), 100 μM GTP (C), or 100 μM GPP(CH)p (D) at 30° for 40 min. Values are means ± standard errors of three determinations. Significant difference from the binding of control membranes in the absence of GTP analog (□), *, $p < 0.05$; **, $p < 0.01$.

the reaction mixture was incubated at 30° for 40 min, it was filtered under vacuum-reduced pressure through a Whatman GF/C filter (presoaked in 0.3% polyethyleneimine) and the filter was rinsed three times with 1.5 ml of ice-cold buffer. Specific binding was defined as the bound radioactivity determined after subtraction of nonspecific binding (in the presence of 10 μM 5-HT for 5-HT_{1A} receptors and 10 μM phentolamine for α_2-adrenoceptors) from total binding. Statistical differences between control and test values were analyzed by Student's *t* test.

Scatchard plots of the equilibrium binding of [³H]8-OH-DPAT (0.05–20 nM) were analyzed by a computerized nonlinear least squares curve-fitting procedure (11), using the method of Rosenthal (16).

Solubilization of [³H]8-OH-DPAT-binding protein and gel filtration. Pretreated membranes were incubated with [³H]8-OH-DPAT (5 nM) in the presence or absence of GTPγS (10 μM) and were washed twice. The radiolabeled membranes were then incubated with 0.7% CHAPS buffer (containing 0.2 mM PMSF in 50 mM Tris·HCl, pH 7.4) for 30 min at 4°. Following this treatment, the mixture was centrifuged at 100,000 × *g* for 30 min. Supernatant thus obtained was used as a solubilized [³H]8-OH-DPAT-binding protein. A column (1 × 25 cm) of Ultrogel AcA 34 was equilibrated and eluted at 4° with 50 mM Tris·HCl buffer (pH 7.4) containing 0.3% CHAPS and 0.2 mM PMSF. The solubilized sample (0.5 ml) was applied to this column and every 15 drops (0.5 ml) were collected. The radioactivity of each fraction (0.4 ml) was counted. The column was calibrated with standard proteins: glyceraldehyde-3-phosphate dehydrogenase (M_r 36,000), BSA (M_r 67,000), and G_o ($\alpha_{39}\beta\gamma$) purified from rat brain (M_r about 80,000).

[³⁵S]GTPγS binding. Pretreated membranes or G_o protein samples were incubated with 1 μM [³⁵S]GTPγS at 30° for 60 min. [³⁵S]GTPγS bound to proteins was determined by rapid filtration (6).

IAP-catalyzed [³²P]ADP-ribosylation of G_o. The ADP-ribosylation by IAP was carried out as described previously (14). Drug-pretreated G_o was incubated with 10 μG [³²P]NAD, 10 μg/ml preactivated IAP, 5 mM ATP, 0.5 mM GTP, 1 mM EDTA, and 10 mM thymidine. After incubation at 30° for 60 min, the reaction was terminated and the radioactivity of [³²P]ADP-ribosylated $G_{o\alpha}$ (M_r 39,000) was determined (17).

Immunoblot assay. Anti-G_o antiserum was prepared and the immunoblot assay was performed as described previously by Kitamura *et al.* (17). Thirty micrograms of proteins from pretreated membranes were dissolved in Laemmli's buffer and subjected to SDS-PAGE (10% polyacrylamide) according to the method of Laemmli (18). Western

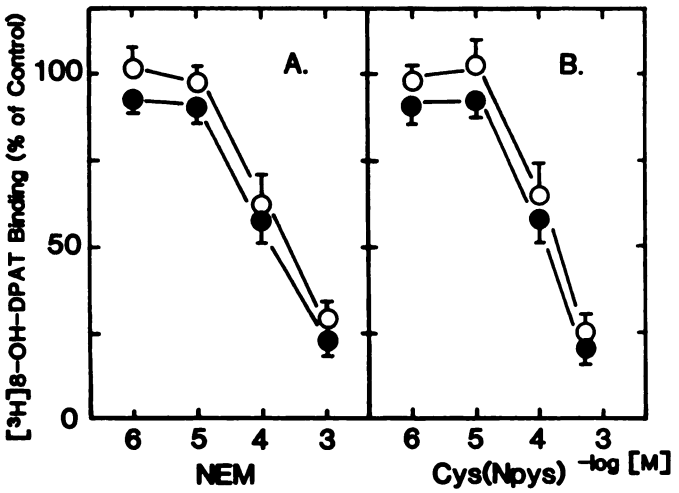

Fig. 5. Effects of NEM and Cys(Npys) on [³H]8-OH-DPAT binding in Boc-Cys(Npys)-pretreated membranes. Treatment with Boc-Cys(Npys) is described in Experimental Procedures. The treated membranes and 0.2 nM [³H]8-OH-DPAT were incubated with increasing concentrations of NEM (A) and Cys(Npys) (B) in the presence (●) or absence (○) of 10 μM GTPγS. Values are means ± standard errors of three determinations.

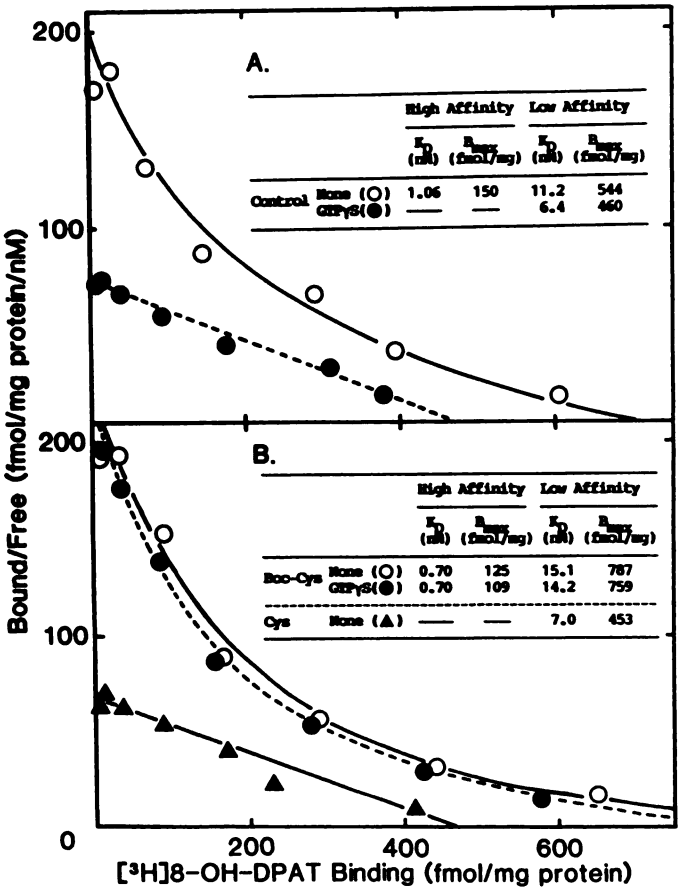

Fig. 6. Changes in Scatchard plot parameters for [³H]8-OH-DPAT binding by GTPγS and Npys compounds. Treatments with Npys compounds are described in Experimental Procedures. Control membranes (A) or Cys(Npys)- (▲) or Boc-Cys(Npys)-pretreated (○, ●) membranes (B) were incubated with various concentrations of [³H]8-OH-DPAT (0.05–20 nM) in the presence (●) or absence (○, ▲) of 10 μM GTPγS. Each point is the mean of duplicate determinations. *Insets*, parameters of [³H]8-OH-DPAT binding analyzed by a computerized nonlinear least squares curve-fitting procedure.

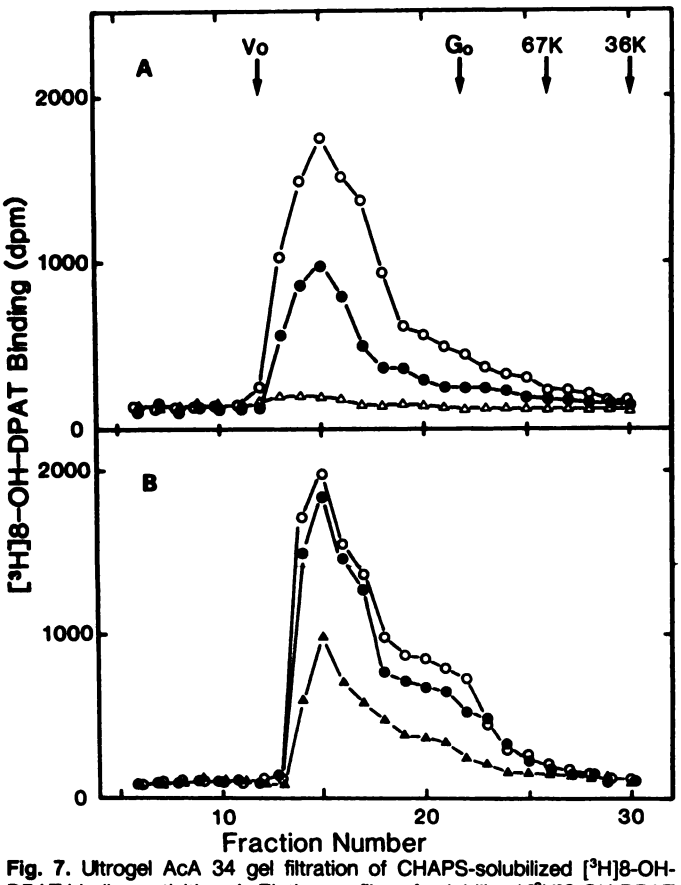

Fig. 7. Ultrogel AcA 34 gel filtration of CHAPS-solubilized [³H]8-OH-DPAT binding activities. A, Elution profiles of solubilized [³H]8-OH-DPAT (5 nM) binding activities of control membranes in the presence of 10 μM GTPγS (●) or 10 μM 5-HT (△) or their absence (○). B, Elution profiles of the binding activities of Cys(Npys)- (▲) or Boc-Cys(Npys)-pretreated membranes (○, ●) in the presence (●) or absence (○, ▲) of 10 μM GTPγS.

blots were performed to transfer proteins from a slab gel to a Durapore GVHP sheet by electroelution at a constant voltage of 30 V at 4° overnight. After the transfer, the sheet was incubated with PBS containing 1% BSA (PBS/BSA) at 4° overnight, to block nonspecific protein binding. The sheet was subsequently incubated with PBS/BSA containing anti-G_o antiserum (17) diluted to 1:200, at 37° for 1 hr. After being rinsed with PBS, the sheet was incubated with a second antibody, horseradish peroxidase-labeled anti-mouse IgG diluted to 1:500, in PBS/BSA at 37° for 1 hr. The sheet was washed with PBS, and then 0.6 mg/ml 4-chloro-1-naphthol and 0.02% H_2O_2 in 10 mM Tris·HCl (pH 7.4) were added. The protein bands that cross-reacted with antibodies against G_o subunits (α_{39}, $\beta_{35/36}$) could be visually detected after 3–5 min at room temperature. The sheet was then washed with water and dried.

Results

Effects of GTP analogs, IAP, and NEM on [³H]8-OH-DPAT binding. Specific [³H]8-OH-DPAT binding to rat brain membranes was decreased by GTP analogs; the order of the inhibitory potency was GTPγS > Gpp(NH)p > GTP ≫ Gpp(CH)p (Fig. 1A). Pretreatment of the membranes with IAP resulted in a concentration-dependent decrease in [³H]8-OH-DPAT binding to the level of binding obtained in the presence of 10 μM GTPγS (Fig. 1B). In addition, NEM pretreatment also decreased binding, similar to the effects of IAP treatment (Fig. 1C). In the presence of GTPγS, however, binding was not changed by either IAP or NEM treatment.

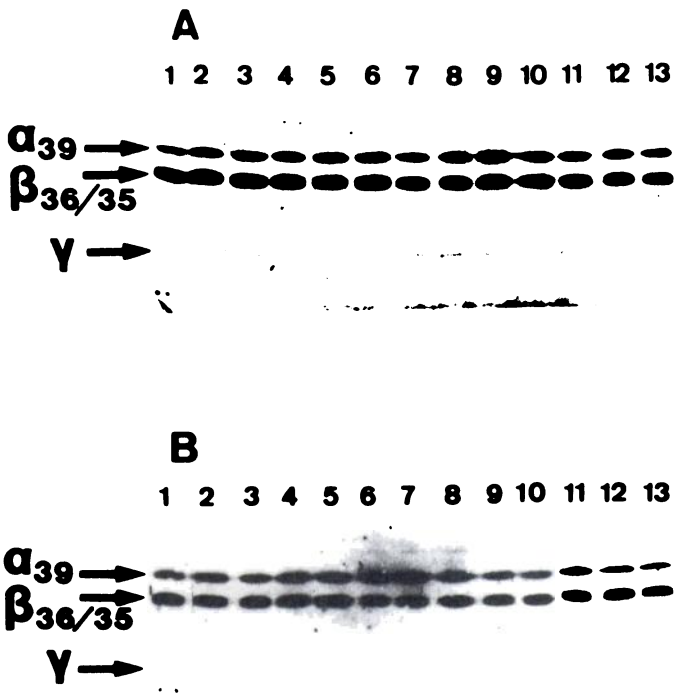

Fig. 8. Effects of Npys compounds on immunoblotting of G_o. The rat brain membranes were treated with vehicle (*lanes 1* and *7*) or 1 μM (*lanes 2* and *8*), 3 μM (*lanes 3* and *9*), 10 μM (*lane 10*), 30 μM (*lanes 4* and *11*), 100 μM (*lanes 5* and *12*), or 300 μM (*lanes 6* and *13*) Cys(Npys) (A) or Boc-Cys(Npys) (B) at 0° for 30 min. Further treatment of these membranes involved incubation with (*lanes 1–6*) or without (*lanes 7–13*) 5 mM DTT. Equal amounts of protein (30 μg) of the treated membranes were loaded onto each lane, and then SDS-PAGE, Western blotting, and immunoblot assay using anti-G_o antiserum were carried out as described in Expeimental Procedures.

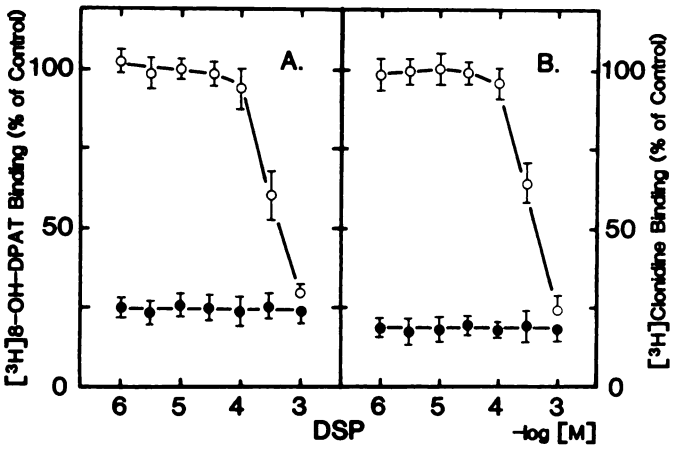

Fig. 9. Effect of DSP pretreatment on [^{3}H]8-OH-DPAT or [^{3}H]clonidine binding. The rat brain membranes were incubated with the indicated concentrations of DSP at 0° for 30 min and then washed, as described in Experimental Procedures. The treated membranes were incubated with 0.2 nM [^{3}H]8-OH-DPAT (A) or 0.5 nM [^{3}H]clonidine (B) at 30° for 40 min, respectively, in the presence (●) or absence (○) of 10 μM GTPγS. Values are means ± standard errors of three determinations.

Effects of pretreatment with Npys compounds on [^{3}H] 8-OH-DPAT and [^{3}H]clonidine binding. Cys(Npys) treatment decreased [^{3}H]8-OH-DPAT and [^{3}H]clonidine binding in a concentration-dependent manner, similar to the effect of IAP or NEM treatment. Treatment with 0.3 mM Cys(Npys) resulted in inhibition of binding of both ligands to the levels obtained in the presence of 10 μM GTPγS (Fig. 2, A and C). Treatment

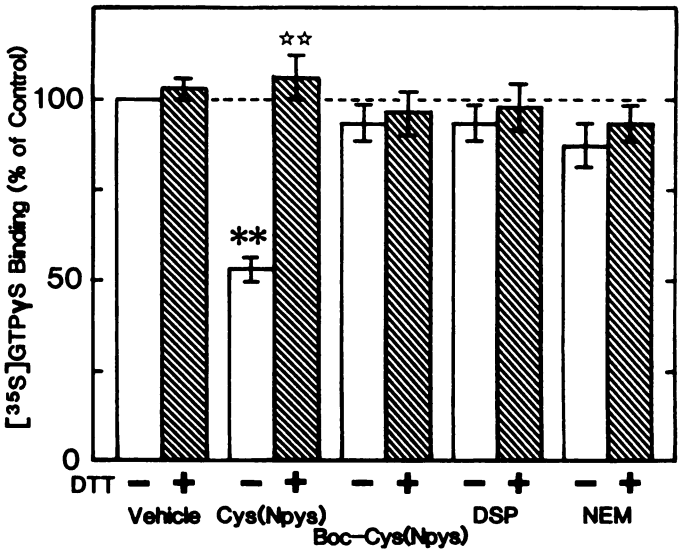

Fig. 10. Effects of Npys compounds, NEM, or DSP pretreatment on [^{35}S] GTPγS binding in the brain membranes. The rat brain membranes were treated with vehicle, 0.3 mM Cys(Npys), 0.3 mM Boc-Cys(Npys), 1 mM DSP or 0.3 mM NEM. The pretreated membranes were further incubated in the presence (▨) or absence (□) of 5 mM DTT, as described in Experimental Procedures. The treated membranes were incubated with 1 μM [^{35}S]GTPγS at 30° for 60 min. Values are means ± standard errors of three determinations. Significance: **, $p < 0.01$ versus vehicle; ☆☆, $p < 0.01$ versus same pretreatment without DTT.

with 1–100 μM Cys(Npys) partially suppressed the inhibitory effect of GTPγS. Further treatment of Cys(Npys)-pretreated membranes with 5 mM DTT completely abolished the inhibitory effect of Cys(Npys) on [^{3}H]clonidine and [^{3}H]8-OH-DPAT binding in the absence of GTPγS and eliminated the disinhibitory effects of low Cys(Npys) concentrations in the presence of GTPγS (Fig. 2, B and D). DTT treatment of NEM-pretreated membranes, however, did not change [^{3}H]clonidine and [^{3}H]8-OH-DPAT binding (data not shown).

On the other hand, Boc-Cys(Npys) treatment did not affect [^{3}H]8-OH-DPAT binding. [^{3}H]Clonidine binding was partially decreased by treatment with Boc-Cys(Npys) at high concentrations (0.1–0.3 mM) in the absence of GTPγS (Fig. 3, A and C). Although [^{3}H]8-OH-DPAT and [^{3}H]clonidine binding to control membranes was decreased in the presence of GTPγS, Boc-Cys(Npys) treatment suppressed the inhibitory effects of GTPγS in both cases in a concentration-dependent manner, and 0.3 mM Boc-Cys(Npys) almost completely blocked the inhibitory effect of GTPγS (Fig. 3, A and C). DTT treatment of Boc-Cys(Npys)-pretreated membranes did not alter [^{3}H]8-OH-DPAT binding in the presence or absence of 10 μM GTPγS (Fig. 3, B and D). However, DTT treatment eliminated the inhibition of [^{3}H]clonidine binding observed at high concentrations of Boc-Cys(Npys) in the absence of GTPγS.

In Boc-Cys(Npys)-pretreated membranes, other guanine nucleotides, such as Gpp(NH)p, GTP, and Gpp(CH)p, did not affect [^{3}H]8-OH-DPAT binding (Fig. 4). Neither DTT nor mercaptoethanol (up to 10 mM) altered [^{3}H]8-OH-DPAT binding, but tri-*n*-butylphosphine alone (over 0.1 mM) inhibited binding (data not shown). In addition, DTT and 2-mercaptoethanol treatment of Boc-Cys(Npys)-pretreated membranes could not reserve the inhibitory effects of GTPγS, Gpp(NH)p, GTP, and Gpp(CH)p on [^{3}H]8-OH-DPAT binding (Fig. 4).

The addition of NEM or Cys(Npys) decreased [^{3}H]8-OH-

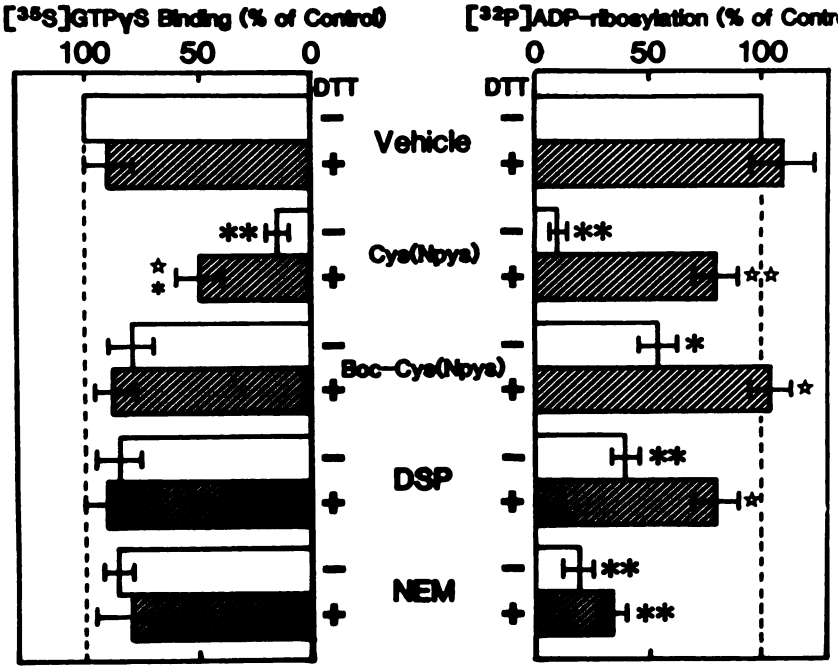

Fig. 11. Effects of Npys compounds, NEM, or DSP on [^{35}S]GTPγS binding or [^{32}P]ADP-ribosylation by IAP in purified G_o. G_o purified from rat brain was pretreated with vehicle, 0.3 mM Cys(Npys), 0.3 mM Boc-Cys(Npys), 1 mM DSP, or 0.3 mM NEM. Then the pretreated G_o sample was further treated with vehicle (□) or 5 mM DTT (▨), as described in Experimental Procedures. Each treated G_o was incubated with 1 μM [^{35}S]GTPγS (*left*) or 10 μM [^{32}P]NAD in the presence of 10 μg/ml IAP (*right*) at 30° for 60 min. Values are means ± standard errors of three determinations. Significant difference: *, $p < 0.05$; **, $p < 0.01$ versus vehicle; ☆, $p < 0.05$; ☆☆, $p < 0.01$ versus same pretreatment without DTT.

DPAT binding to Boc-Cys(Npys)-pretreated membranes in a concentration-dependent manner, and, at concentrations of 1 mM, both reagents reduced binding to levels obtained in control membranes in the presence of GTP analog (Fig. 5).

The equilibrium binding of [^{3}H]8-OH-DPAT (0.05–20 nM) yielded an upward curvilinear Scatchard plot, similar to that obtained for [^{3}H]clonidine binding (11). Analysis by a two-affinity state model provided a better fit to the data than a one-affinity state model. The addition of 10 μM GTPγS, however, resulted in a linear Scatchard plot for [^{3}H]8-OH-DPAT binding, which is best analyzed by a model for a single state, i.e., a low affinity site (Fig. 6A). In the Boc-Cys(Npys)-treated membranes, [^{3}H]8-OH-DPAT binding also showed an upward curvilinear plot in the presence or absence of GTPγS, indicating high and low affinity sites for which B_{max} was not altered in the presence or absence of GTPγS. In the Cys(Npys)-treated membranes, however, binding followed a linear plot, similar to that obtained for binding in the presence of GTPγS in control membranes (Fig. 6B).

Effects of GTPγS and Npys compounds on solubilized [^{3}H]8-OH-DPAT-binding proteins. The CHAPS-solubilized [^{3}H]8-OH-DPAT-binding proteins were gel-filtered into an Ultrogel AcA 34 column (1 × 25 cm). Fig. 7A shows a typical elution profile for the binding proteins in the presence or absence of GTPγS or 5-HT. Solubilized [^{3}H]8-OH-DPAT binding was observed in fractions that appeared to be larger in molecular size than G_o (about 80,000). [^{3}H]8-OH-DPAT-binding activities were inhibited by GTPγS and were completely blocked by 5-HT. The elution profiles of [^{3}H]8-OH-DPAT-binding activities of preparations solubilized from Boc-Cys(Npys)-pretreated membranes were similar in the presence or absence of GTPγS. However, the profile of binding activities of preparations solubilized from Cys(Npys)-pretreated membranes was similar to the profile observed in control membranes in the presence of GTPγS (Fig. 7B).

Effects of pretreatment with Npys compounds on immunoblotting of G_o. Because DTT and mercaptoethanol could not reverse the effects of GTP analogs in Boc-Cys(Npys)-pretreated membranes, we next tried to determine whether modification by Boc-Cys(Npys) is an irreversible cross-linking or an affinity binding. Mobility and density of G_o subunits were determined by SDS-PAGE, employing an antiserum against G_o purified from procine brain (17) that recognized α_{39} and $\beta_{36/35}$ subunits but recognized γ subunits only weakly (Fig. 8). Neither Cys(Npys) nor Boc-Cys(Npys) affected either mobility or densities of the subunits of G_o (α_{39}, $\beta_{36/35}$, and γ) (Fig. 8). Treatment with DTT had no effect.

Effects of pretreatment with DSP on [^{3}H]clonidine and [^{3}H]8-OH-DPAT binding. It has been reported that DSP causes a cross-linking between PGE_2 receptors and IAP-insensitive G proteins, which results in an abolition of the GTP effect on [^{3}H]PGE_2 binding (14). In this experiment, DSP treatment did not affect [^{3}H]8-OH-DPAT and [^{3}H]clonidine binding at low concentrations (~0.1 mM) and decreased binding at high concentrations (0.3–1 mM), similar to the inhibitory effects of NEM and Cys(Npys) (Fig. 9). In the presence of GTPγS, DSP did not affect binding of either ligand.

Effects of Npys compounds, NEM, and DSP on [^{35}S]GTPγS binding and IAP-catalyzed [^{32}P]ADP-ribosylation in purified G_o. The findings described above suggested that these reagents may modify G proteins. In the membranes treated with 0.3 mM Cys(Npys), [^{35}S]GTPγS binding was about 50% of that in control membranes and further treatment of Cys(Npys)-pretreated membranes with DTT led to a recovery of binding to the level of control membranes (Fig. 10). Other reagents did not affect the binding.

Our previous studies demonstrated that α_2-adrenoceptors (11) and 5-HT_{1A} receptors (4) coupled with IAP-sensitive G proteins (G_i/G_o). Therefore, we examined the effects of Npys compounds, NEM, and DSP on [^{35}S]GTPγS binding and IAP-catalyzed [^{32}P]ADP-ribosylation in purified G_o from rat brain. In G_o, Cys(Npys) treatment inhibited [^{35}S]GTPγS binding to about 80% of control, but other reagents had only slight inhibitory effects (Fig. 11). DTT treatment of Cys(Npys)-pretreated

G_o suppressed the inhibition of binding to a level of 50% of control binding but did not change the effects of other pretreatments. In addition, Cys(Npys), NEM, and DSP markedly inhibited the IAP-catalyzed [^{32}P]ADP-ribosylation of purified G_o, but Boc-Cys(Npys) resulted in inhibition of only approximately 50% of control activity (Fig. 11). Secondary treatment with DTT led to a near recovery of activity in G_o pretreated with Cys(Npys), Boc-Cys(Npys), and DSP.

Discussion

IAP and NEM cause uncoupling of an inhibitory receptor from G protein, resulting in a decrease of agonist binding to α_2-adrenoceptors (1, 2) and 5-HT_{1A} receptors (Fig. 1). IAP catalyzes on ADP-ribosylation of a cysteine residue located at the fourth position from the C-terminus of the α-subunit of several G proteins, i.e., G_i ($\alpha_{41}\beta\gamma$), G_o ($\alpha_{39}\beta\gamma$), $\alpha_{40}\beta\gamma$, and transducin, resulting in the uncoupling of receptors from G proteins (8, 19, 20). In addition, NEM treatment of brain membranes and purified G proteins inhibits IAP-catalyzed ADP-ribosylation, suggesting that NEM irreversibly alkylates a cysteine residue(s) that is the same as or near the cysteine targeted for ADP-ribosylation by IAP on α-subunits of G proteins (10, 11, 21). In this study, both [^{3}H]8-OH-DPAT and [^{3}H]clonidine binding were decreased by one Npys compound, Cys(Npys), in a way similar to the effects of NEM and IAP. Cys(Npys) treatment also inhibited the IAP-catalyzed ADP-ribosylation of purified G_o, again following the behavior of NEM. These results appear to indicate that Cys(Npys) modifies a cysteine residue(s) that is the same as the cysteine residue(s) alkylated by NEM and that is or is near the cysteine of ADP-ribosylation by IAP in α-subunits of G proteins, and this compound causes an uncoupling of receptors from IAP-sensitive G proteins. In addition, Cys(Npys) inhibited [^{35}S]GTPγS binding to brain membranes and purified G_o, indicating that this reagent also modifies a cysteine residue(s) that is on or near the GTP-binding domain of G proteins. Cys(Npys) (1–100 μM) partially inhibited GTPγS effects on agonist binding, suggesting that, at these concentrations, Cys(Npys) may modify this cysteine residue(s) (Fig. 2, A and C). However, these effects were completely reversed by treatment with a reducing agent (DTT).

Boc-Cys(Npys) treatment abolished the inhibitory effects of GTP analogs [GTPγS, Gpp(NH)p, GTP, and Gpp(CH)p] on [^{3}H]8-OH-DPAT and [^{3}H]clonidine binding, which were recovered by treatment with some reducing reagents, although neither DTT nor mercaptoethanol had this recovery effect. Scatchard plots of [^{3}H]8-OH-DPAT binding indicated the presence of two affinity states, high and low (Fig. 6). GTPγS and Cys(Npys) treatment gave data that fit a single-low affinity state model. However, results obtained after Boc-Cys(Npys) treatment indicated two affinity binding states in either the presence or the absence of GTPγS. For agonist binding, it is known that a high affinity state consists of a ternary agonist·receptor·G protein ($A \cdot R \cdot G$) complex, whereas a low affinity state comprises a binary agonist·receptor ($A \cdot R$) complex (1, 4, 11). Although the 5-HT_{1A} receptor has yet to be purified, recent determination of the primary amino acid sequence structure (22, 23) by hybridization of a β_2-adrenoceptor probe indicates that it is a member of a superfamily of G protein-coupling receptors. It is known that G protein-coupling receptors are capable of *N*-linked glycosylation, possess seven membrane-spanning domains, and have a molecular weight of 50,000–80,000 (24). However, the elution profiles of solubilized [^{3}H]8-OH-DPAT-binding proteins showed larger molecular weights than G_o (about 80,000) (Fig. 7), indicating that this major peak of solubilized [^{3}H]8-OH-DPAT-binding proteins may exist in the coupling form of $A \cdot R \cdot G$.

The peak of [^{3}H]8-OH-DPAT binding activity in Cys(Npys)-pretreated membranes was lower than that in vehicle and similar to control in the presence of GTPγS. In contrast, the profiles of [^{3}H]8-OH-DPAT binding activities of Boc-Cys(Npys)-pretreated membranes were similar in the presence or absence of GTPγS, suggesting that Boc-Cys(Npys) seems to lock a coupling of receptor and G protein ($R \cdot G$). Boc-Cys(Npys), however, could not prevent the NEM- or Cys(Npys)-induced uncoupling of the receptor from G proteins (Fig. 5). Although Boc-Cys(Npys) pretreatment abolished the GTP effect on agonist binding in preparations further treated by DTT and mercaptoethanol, Boc-Cys(Npys) did not affect the mobility or the density of G_o subunits in SDS-PAGE (Fig. 8). These results seem to indicate that the modifying effect of Boc-Cys(Npys) is based on a conformational change and/or a hydrophobic binding [the butoxycarbonyl residue of Boc-Cys(Npys)] between receptors and G proteins, rather than an irreversible covalent cross-linking. Thus, Boc-Cys(Npys) induces stabilization of receptor coupling with G proteins, resulting in elimination of the inhibitory effects of GTP analogs. In addition, Boc-Cys(Npys) did not inhibit the GTPγS effect on isoproterenol competition of ^{125}I-cyanopindolol binding to β-adrenoceptors (data not shown). It is known that stimulation of α_2-adrenoceptors (2) and 5-HT_{1A} receptors (25) inhibits adenylate cyclase activity and that the stimulation of β-adrenoceptors activates adenylate cyclase via G_s (26). It appears that Boc-Cys(Npys) stabilizes the coupling of inhibitory receptors with IAP-sensitive G proteins.

At high concentrations (over 0.1 mM), Boc-Cys(Npys) inhibited [^{3}H]clonidine binding but did not suppress [^{3}H]8-OH-DPAT binding (Fig. 3, A and C). The inhibitory effect was completely canceled by further DTT treatment. We previously reported that NEM at high concentrations alkylated the [^{3}H]clonidine binding domain of the α_2-adrenoceptor, resulting in a decrease of B_{max} of [^{3}H]clonidine binding (11). Regan *et al.* (27), using purified α_2-adrenoceptors from human platelets, found that exposure of the receptors to a sulfhydryl reagent results in loss of the binding activity. Thus, α_2-adrenoceptors appear to require an essential sulfhydryl residue in the binding domain for agonist binding, whereas 5-HT_{1A} receptors do not.

Korner *et al.* (28) reported that treatment with a hormone plus NEM causes the hormone to be locked in the β-adrenoceptor·G_s complex, but NEM alone has no effect. Although the effect of Boc-Cys(Npys) is similar to the NEM effect in the β-adrenoceptor system, Boc-Cys(Npys) by itself leads to stabilization of coupling between inhibitory receptors and IAP-sensitive G proteins. In addition, a recent paper indicates that treatment with DSP induces covalent cross-linking of PGE_2 receptors with IAP-insensitive G proteins and results in inhibition of GTP-induced dissociation of bound [^{3}H]PGE_2 in bovine adrenal medulla (12). In the present study, DSP decreased [^{3}H]8-OH-DPAT and [^{3}H]clonidine binding, similar to the effects of IAP, NEM (1, 9), and Cys(Npys). These results suggest that the inhibitory receptor system with IAP-sensitive

G protein is different from that of the PGE_2 receptor with IAP-insensitive G protein.

In conclusion, Npys compounds, Cys(Npys) and Boc-Cys(Npys), appear to be unique and interesting drugs to be used as sulfhydryl-modifying reagents for studies of the receptor-G protein coupling system.

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