0026-895X/86/030244-06$02.00/0

MOLECULAR PHARMACOLOGY, 29:244-249

Uncoupling of γ-Aminobutyric Acid B Receptors from GTP-Binding Proteins by *N*-Ethylmaleimide: Effect of *N*-Ethylmaleimide on Purified GTP-Binding Proteins

TOMIKO ASANO and NOBUAKI OGASAWARA

Department of Biochemistry, Institute for Developmental Research, Aichi Prefectural Colony, Kasugai, Aichi 480-03, Japan

Received August 8, 1985; Accepted December 13, 1985

SUMMARY

Treatment of membranes from bovine cerebral cortex with *N*-ethylmaleimide (NEM) resulted in inhibition of γ-aminobutyric acid (GABA) binding to $GABA_B$ receptors. The binding curve for increasing concentrations of agonist was shifted to the right by NEM treatment. Guanine nucleotide had little effect on the binding of GABA to NEM-treated membranes. The addition of purified GTP-binding proteins, which were the substrates of islet-activating protein (IAP), pertussis toxin, to the NEM-treated membranes caused a shift of the binding curve to the left, suggesting modification of GTP-binding proteins rather than receptors by NEM. Therefore, the effect of NEM on two purified GTP-binding proteins, Gi (composed of three subunits with molecular weight of α,41,000; β,35,000; γ,10,000) and Go (α,39,000; β,35,000; γ,10,000) was studied. NEM did not significantly change guanosine 5′-(3-*O*-thio)triphosphate (GTPγS) binding and GTPase activity of these two proteins. In contrast, NEM-treated Gi and Go were not ADP-ribosylated by IAP and did not increase GABA binding to NEM-treated membranes. When α and βγ subunits were treated with NEM and then mixed with nontreated α and βγ to form Gi or Go, respectively, both oligomers with NEM-treated α-subunits lost their abilities to be IAP substrates and to couple to receptors. These results indicate that NEM uncoupled GTP-binding proteins from receptors by modifying α-subunits of GTP-binding proteins, and the site seemed to be on or near the site of ADP-ribosylation by IAP. When α and βγ subunits were treated with NEM and then mixed to form Gi or Go, GTPγS binding in the absence of Mg^{2+} and GTPase activity were changed, although they were not affected when oligomers were treated with NEM. The results suggest the existence of another sulfhydryl group which is protected from NEM by the association of subunits. The modification of this sulfhydryl group by NEM appeared to interfere with the interaction between α and βγ.

The binding of agonists to receptors that are linked to adenylate cyclase via GTP-binding proteins is regulated by guanine nucleotide; the addition of guanine nucleotide causes a transition of the agonist high affinity form to a low affinity form (1). Low concentrations of NEM, a sulfhydryl alkylating agent, have been found to mimic the effect of nucleotides on agonist binding with several receptors, including muscarinic cholinergic (2, 3), D-2 dopamine (4, 5), opiate (6), and adenosine receptors (7). Agonist binding to these receptors in NEM-treated preparations shows low affinity and is insensitive to guanine nucleotide. It also has been reported that α-adrenergic (8) or muscarinic cholinergic (2) receptor-mediated inhibition of adenylate cyclase is abolished by NEM. Since these receptors are believed to be coupled with the inhibitory GTP-binding protein of adenylate cyclase (Gi), NEM seems to modify Gi, but at a site that is unknown.

IAP, pertussis toxin, induces ADP-ribosylation of Gi in parallel with the attenuation of receptor-mediated inhibition of adenylate cyclase activity (9). ADP-ribosylation of Gi by IAP causes uncoupling of Gi from receptors (10–13). The effect of guanine nucleotide on agonist binding to receptor is lost or reduced by treatment of membrane-donor cells with IAP; in the absence of the nucleotide, the affinity of agonist is lower in toxin-treated than in nontreated membrane preparations (10, 11, 13). These observations are very similar to those with NEM.

Sternweis and Robishaw (14) and Neer *et al.* (15) have demonstrated the existence of two Gi-like proteins in brain. One is composed of three subunits with molecular weights of 41,000 (α), 35,000 (β), and 10,000 (γ), and seems to be identical to Gi (termed Gi). The other is also composed of three subunits with molecular weights of 39,000 (α), 35,000 (β), and 10,000

This work was supported in part by Research Grant 60580143 from the Ministry of Education, Science and Culture, Japan.

ABBREVIATIONS: NEM, *N*-ethylmaleimide; IAP, islet-activating protein; Gi, inhibitory GTP-binding protein of the adenylate cyclase system; Go, a new GTP-binding protein from membranes of bovine brain; GABA, γ-aminobutyric acid; GTPγS, guanosine 5′-(3-*O*-thio)triphosphate; Gpp(NH)p, guanosine 5′-(β,γ-imido)triphosphate; DTT, dithiothreitol; Hepes, 4-2(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EDTA, ethylenediaminetetraacetate.

(γ), and was termed Go by Sternweis and Robishaw (14). The α-subunits of both proteins bind GTP and are ADP-ribosylated by IAP. The β- and γ-subunits of both proteins seem to be identical (14).

The GABA and $GABA_B$ receptor agonist, baclofen, inhibits basal adenylate cyclase activity (16) and stimulates GTPase activity (17) in membrane preparations from rat cerebellum. Agonist binding to the $GABA_B$ receptor is inhibited by guanine nucleotide (18, 19). Our previous study demonstrated that IAP causes inhibition of GABA binding by lowering the affinity for receptor, and the addition of purified GTP-binding protein to IAP-treated membranes restores high affinity binding (19). Both Gi and Go could increase GABA binding. These results indicate that $GABA_B$ receptors are coupled to IAP-sensitive GTP-binding proteins.

In this study, we have found a reduction of the affinity of $GABA_B$ receptors for an agonist by NEM treatment of membranes and its restoration by the addition of purified GTP-binding proteins. Because these findings indicated the modification of GTP-binding proteins rather than receptors, the effect of NEM on purified GTP-binding proteins, Gi and Go, was investigated.

Materials and Methods

[2,3-^{3}H]GABA, [adenosine-2,8-^{3}H]NAD, [^{35}S]GTPγS, and [γ-^{32}P]GTP were purchased from New England Nuclear (Boston, MA). IAP was a generous gift of Dr. Michio Ui, Hokkaido University, Sapporo, Japan. Isoguvacine was kindly supplied by Dr. P. Krogsgaard-Larsen, Royal Danish School of Pharmacy, Copenhagen, Denmark. (±)-Baclofen was a gift from Ciba-Geigy (Japan) Ltd.

Membrane preparation. Fresh bovine cerebral cortices were homogenized with 4 volumes of 10 mM Tris-HCl (pH 7.5), 10% sucrose, 0.5 mM phenylmethylsulfonyl fluoride using a Polytron homogenizer. The homogenate was centrifuged at 1,000 × *g* for 10 min to obtain the supernatant, which was further centrifuged at 20,000 × *g* for 30 min. The pellet thus obtained was suspended in the same medium by means of a Potter-Elvehjem homogenizer and sedimented by centrifugation at 20,000 × *g* for 30 min. The sedimented membrane fraction was stored at −80° and used for both [^{3}H]GABA binding and purification of the GTP-binding protein.

Treatment of membranes with NEM. Frozen membranes were thawed, washed once with 50 mM Tris-HCl (pH 8.0), and frozen again at −80° for at least 16 hr. The membranes were then thawed and resuspended in 50 mM Tris-HCl (pH 8.0) (2 mg of protein/ml), and Triton X-100 was added to a final concentration of 0.02%. The suspension was incubated at 37° for 20 min before centrifugation for 10 min at 20,000 × *g*, and the pellet was suspended in 50 mM Tris-HCl (pH 8.0) (4 mg of protein/ml). NEM was added to this suspension to a final concentration of 0.2 mM unless shown otherwise. After incubating for 30 min at 0°, 1 mM DTT was added to the membranes, and then the membranes were centrifuged at 10,000 × *g* for 5 min. The pellet was washed three times by centrifugation to provide the NEM-treated membrane preparation. For nontreated membranes, the membranes were prepared by the same procedure except that 1 mM DTT was added to the membranes before the addition of 0.2 mM NEM. Protein in the membrane preparations was determined by the method of Lowry *et al.* (20).

Purification of GTP-binding proteins. The GTP-binding proteins (mixture of Gi and Go) were purified from bovine cortical membranes according to the method of Sternweis and Robishaw (14). Gi, Goα, and $\beta\gamma$ subunits were resolved according to the method previously described (19). An equal amount of $\beta\gamma$ was added to Goα preparation to form Go. The resolution of Giα from $\beta\gamma$ was performed by heptylamine-Sepharose chromatography as described (21). Final preparations were in 10 mM Hepes (pH 8.0), 1 mM EDTA, 1 mM DTT, and 0.1 or 0.05% Lubrol PX. Protein in the GTP-binding protein preparation was assayed by the method of Schaffner and Weissmann (22).

NEM treatment of GTP-binding protein. Since the preparations of purified GTP-binding proteins contained 1 mM DTT, they were applied to a Sephadex G-25 column equilibrated with 20 mM Hepes (pH 8.0), 1 mM EDTA, and 0.05% Lubrol PX at 4°. The void fraction was divided into two portions. NEM (0.2 mM) was added to one (NEM-treated), and 1 mM DTT was added to the other before the addition of 0.2 mM NEM (nontreated). After 30 min incubation at 0°, 1 mM DTT was added only to the NEM-treated preparation. Then, GTP-binding protein was applied to a Sephadex G-25 column and was eluted with 20 mM Hepes (pH 8.0), 1 mM EDTA, 1 mM DTT, and 0.05% Lubrol. The void fraction was used for various experiments.

Other methods. Binding of [^{3}H]GABA was measured as described previously, except that 50 μM isoguvacine was used to block $GABA_A$ receptor binding (19). Binding of [^{35}S]GTPγS was measured essentially as described by Northup *et al.* (23). ADP-ribosylation by IAP was carried out according to the method of Bokoch *et al.* (24) with 2 μM [^{3}H]NAD. GTPase activity was determined as described by Brandt *et al.* (25) with modification.

Results

Effect of NEM treatment of membranes on GABA binding. Bovine cerebral cortical membranes were incubated with various concentrations of NEM for 30 min at 0° and then washed several times with buffer and subjected to a GABA binding assay. Fig. 1 shows a dose-related decrease of [^{3}H]GABA binding caused by NEM treatment.

To test whether this NEM effect was due to modification of $GABA_B$ receptor protein or GTP-binding proteins coupled to the receptor, the effect of guanine nucleotide on GABA binding was examined. Fig. 2 shows [^{3}H]GABA binding to nontreated or NEM-treated membranes in the presence or absence of 50 μM Gpp(NH)p. Binding measurements were not feasible above 300 nM GABA because of high nonspecific binding. Although saturation kinetics was not observed, the binding curve was

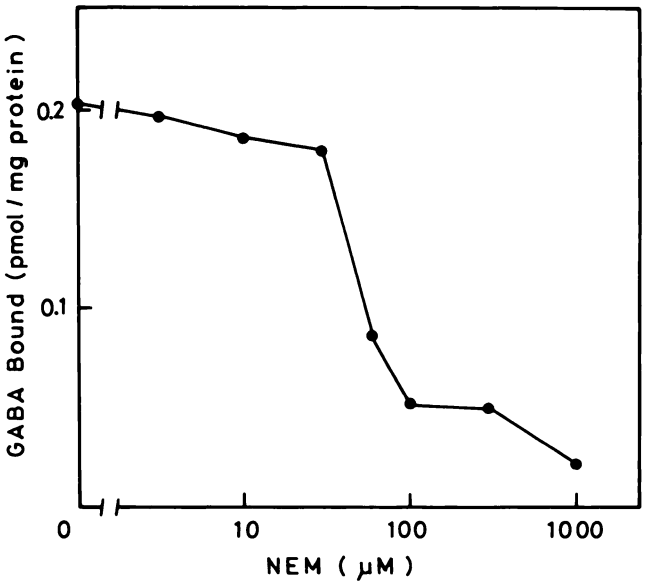

Fig. 1. Effect of NEM treatment of membranes on GABA binding to $GABA_B$ receptor. The membranes (4 mg of protein/ml) were incubated with the indicated concentrations of NEM for 30 min at 0°. At the end of the incubation, 1 mM DTT was added to the reaction mixture, which was then centrifuged, and the pellet was washed as described in Materials and Methods. [^{3}H]GABA binding was measured at a GABA concentration of 10 nM.

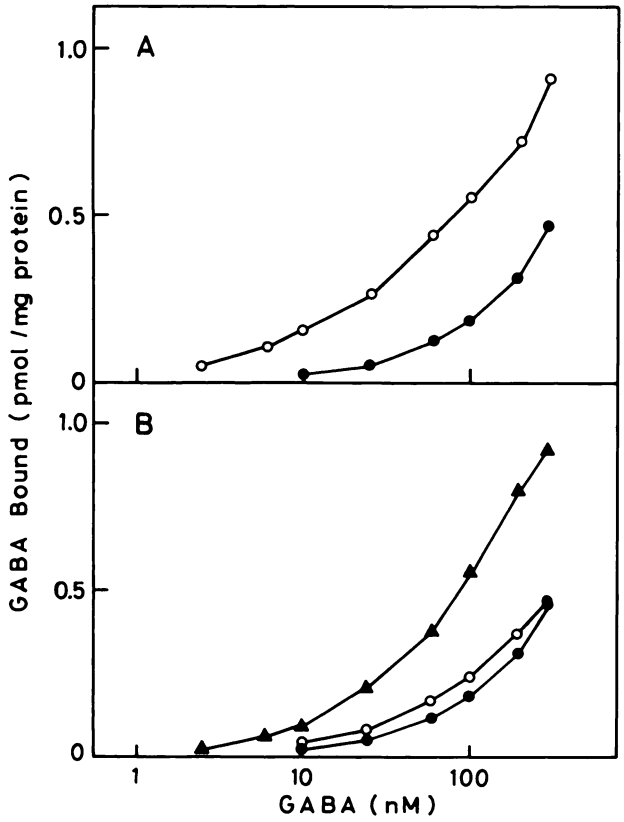

Fig. 2. [^{3}H]GABA binding to NEM-treated or nontreated membranes in the presence or absence of Gpp(NH)p and the effect of GTP-binding protein on the binding to NEM-treated membranes. [^{3}H]GABA binding to nontreated (A) or NEM-treated (B) membranes was determined with increasing concentrations of GABA in the presence (●) or absence (○) of 50 μM Gpp(NH)p. GTP-binding proteins (mixture of Gi and Go, 50 pmol or 5 μg) were incubated with NEM-treated membranes (0.15–0.2 mg of protein) for 15 min at 0°, and then [^{3}H]GABA binding was measured (▲). All reaction mixtures included 0.02% cholate.

shifted to the right by the addition of the nucleotide to nontreated membranes, suggesting a decrease of the affinity of agonist as described previously (19). In the case of NEM-treated membranes, the binding curve was shifted to the right, and Gpp(NH)p slightly affected it. In the presence of Gpp(NH)p, the binding curve of NEM-treated membranes was essentially identical to the curve of nontreated membranes. When purified GTP-binding proteins (mixture of Gi and Go) were added to NEM-treated membranes, the binding curve was shifted to the left, suggesting restoration of high affinity binding (Fig. 2). These results indicate that the decrease of GABA binding by NEM treatment was due to modification of GTP-binding proteins rather than $GABA_B$ receptors.

The effect of NEM on purified GTP-binding proteins, Gi and Go. First, the effect of NEM on GTPγS binding and GTPase activity of Gi and Go was determined. It was observed that NEM did not significantly change GTPγS binding or GTPase activity of GTP-binding proteins. These results suggest that NEM did not affect the GTP-binding site.

NEM-treated or nontreated GTP-binding proteins were subjected to ADP-ribosylation by IAP *plus* [^{3}H]NAD. Table 1 shows that NEM-treated Gi and Go were not ADP-ribosylated at all. When the ability of NEM-treated or nontreated GTP-binding proteins to increase $GABA_B$ receptor binding to NEM-treated membrane was measured, both nontreated Gi and Go

TABLE 1

Effect of NEM on ADP-ribosylation of GTP-binding proteins by IAP and the ability of these proteins to increase GABA binding

Purified Gi, Go, and their subunits were treated with NEM as described under Materials and Methods. An equal amount of βγ was added to Giα or Goα to form Gi or Go after NEM treatment. GTP-binding proteins (1 μg) were subjected to ADP-ribosylation by IAP. For GABA binding, GTP-binding proteins (50 pmol, 4.8–6.8 μg) were added to NEM-treated membranes (0.1–0.2 mg of protein) and incubated at 0° for 15 min. The GABA binding assay was then started by the addition of reaction mixture. The [^{3}H]GABA concentration was 30 nM, and the final concentration of Lubrol was 0.005%. Basal binding (without GTP-binding protein) was 93 ± 11 fmol/mg of protein. The data are shown as the binding with GTP-binding protein minus basal binding (means ± standard error of three to five experiments).

GTP-binding protein	Treatment		ADP-ribosylation	Increase of GABA binding
			nmol/mg protein	*fmol/mg protein*
Gi (oligomer)	none		4.66 ± 0.08	184 ± 10
	NEM		0.09 ± 0.04	19 ± 7
Go (oligomer)	none		4.56 ± 0.54	140 ± 10
	NEM		0.02 ± 0.01	19 ± 11
	α	βγ		
Gi (subunit)	none	none	4.29 ± 0.29	199 ± 22
	NEM	none	0.28 ± 0.14	22 ± 11
	none	NEM	3.83 ± 0.52	167 ± 33
	NEM	NEM	0.22 ± 0.16	5 ± 2
Go (subunit)	none	none	4.15 ± 0.70	138 ± 8
	NEM	none	0.12 ± 0.06	21 ± 14
	none	NEM	3.89 ± 0.57	115 ± 4
	NEM	NEM	0.05 ± 0.03	16 ± 11

increased GABA binding, but NEM-treated GTP-binding proteins did not significantly change the binding (Table 1). To examine which subunit of GTP-binding protein was modified by NEM, α- and βγ-subunits were treated with NEM, and NEM-treated and nontreated subunits were mixed with a ratio of α to βγ of 1:1 to form Gi or Go. These GTP-binding proteins were subjected to ADP-ribosylation by IAP, or their abilities to increase GABA binding to NEM-treated membranes were determined. Table 1 shows that both Gi and Go with NEM-treated α-subunits could not be IAP substrates and did not increase GABA binding. The effect of NEM, to prevent GTP-binding proteins from ADP-ribosylation by IAP, was parallel with its effect to prevent GTP-binding proteins to couple with receptors.

Further effect of NEM on the subunits of GTP-binding proteins. When Gi or Go oligomer was treated with NEM, GTPγS binding and GTPase activity did not significantly change (Table 2). However, when each α-subunit was treated with NEM and then an equal amount of NEM-treated βγ was added to form Gi or Go, both GTPγS bindings in the absence of Mg^{2+} were increased (Table 2), although binding in the presence of $MgCl_2$ was not affected (data not shown). The change of GTPγS binding observed with Gi was not significant, but it tended to be increased by NEM. Conversely, GTPase activity observed with Gi subunits in the presence of 20 mM $MgCl_2$ was decreased by NEM treatment, whereas GTPase activity observed with Go subunits in the presence of 2 mM $MgCl_2$ was increased by it. The results suggest that there was another sulfhydryl group on α- or βγ-subunits and the association of subunits protected it from NEM.

Fig. 3 shows the time course of association of GTPγS with NEM-treated or nontreated Goα in the absence of Mg^{2+}. The bindings to both preparations of Goα were roughly identical. Each binding was reversed by the addition of GTP. The addition of βγ-subunits to nontreated α that had reached an equilibrium level of binding caused a rapid and marked reversal of

TABLE 2

Effect of NEM treatment of oligomers or subunits of GTP-binding proteins on GTPγS binding and GTPase activity

Gi, Go, and their subunits were treated with NEM as described under Materials and Methods. An equal amount of βγ was added to α to form Gi or Go after NEM treatment. GTPγS binding was measured in the reaction mixture containing 1 μM [^{35}S]GTPγS, 20 mM Hepes (pH 8.0), 1 mM EDTA, 1 mM DTT, 0.05% Lubrol PX. Incubation was carried out at 30° for 60 min (Gi) or 15 min (Go). The GTPase assay was performed at 30° for 10 min in the reaction mixture containing 1 μM [γ-^{32}P] GTP, 50 mM Hepes (pH 8.0), 1 mM EDTA, 1 mM DTT, 0.1 M NaCl, 0.1 mM adenyl-5′-yl imidodiphosphate, 0.05% Lubrol PX, and 20 mM $MgCl_2$ for Gi or 2 mM $MgCl_2$ for Go. The data are presented as means ± standard error of three to eight experiments.

GTP-binding protein	Treatment		GTPγS binding	GTPase
			nmol/mg protein	*nmol/min/mg protein*
Gi (oligomer)	none		3.89 ± 0.35	1.43 ± 0.13
	NEM		3.48 ± 0.32	1.39 ± 0.16
Go (oligomer)	none		5.31 ± 0.38	2.18 ± 0.33
	NEM		5.15 ± 0.26	2.15 ± 0.28
	α	βγ		
Gi (subunit)	none	none	3.99 ± 0.32	1.48 ± 0.10
	NEM	NEM	4.58 ± 0.42	1.18 ± 0.08[a]
Go (subunit)	none	none	5.51 ± 0.47	2.23 ± 0.24
	NEM	NEM	7.12 ± 0.46[a]	3.75 ± 0.31[b]

[a] Significantly different ($p < 0.05$).
[b] Significantly different ($p < 0.01$).

this binding; the amount of bound GTPγS was reduced to the level observed when βγ was included initially. In contrast, the addition of βγ to NEM-treated α caused only a small reduction of this binding. NEM-treated βγ had the same ability to reverse the binding as did nontreated βγ (data not shown).

The effect of βγ on GTPγS binding to NEM-treated or nontreated Goα is shown in Fig. 4. The binding to nontreated Goα showed a concentration-dependent decrease upon the addition of βγ, whereas the binding to NEM-treated α was only slightly decreased with βγ.

The effect of βγ on GTPase activity was examined with nontreated or NEM-treated α (Fig. 5). The addition of βγ to Giα caused an increase in GTPase activity when the activity was measured in the presence of 20 mM $MgCl_2$. The activity of NEM-treated Giα, however, was not altered by the addition of βγ (Fig. 5A). In contrast, the addition of βγ to Goα caused a decrease in GTPase activity when the activity was measured in the presence of 2 mM $MgCl_2$ (Fig. 5B). NEM-treated Goα showed much higher levels of GTPase activity than did nontreated α, and the activity also was decreased by the addition of βγ, but the extent of the inhibition caused with the same amount of βγ was much smaller in the case of NEM-treated α. NEM-treated βγ showed equal ability to nontreated βγ in decreasing the GTPase activity of Goα (Fig. 5B).

Discussion

An NEM-induced decrease in the affinity of receptor for agonist and a loss of sensitivity to guanine nucleotide have been noted for various receptors (2–7). As these receptors are believed to be coupled to Gi, NEM seems to uncouple them from Gi. In our previous paper, we showed coupling of Gi and Go to the $GABA_B$ receptor (19). Therefore, it was expected that the $GABA_B$ receptor also would be uncoupled by NEM from GTP-binding proteins as with other receptors. Our study shows that the effect of NEM on the $GABA_B$ receptor is the same as that found with other receptors: loss of sensitivity for guanine nucleotide and decrease in agonist affinity. The restoration of

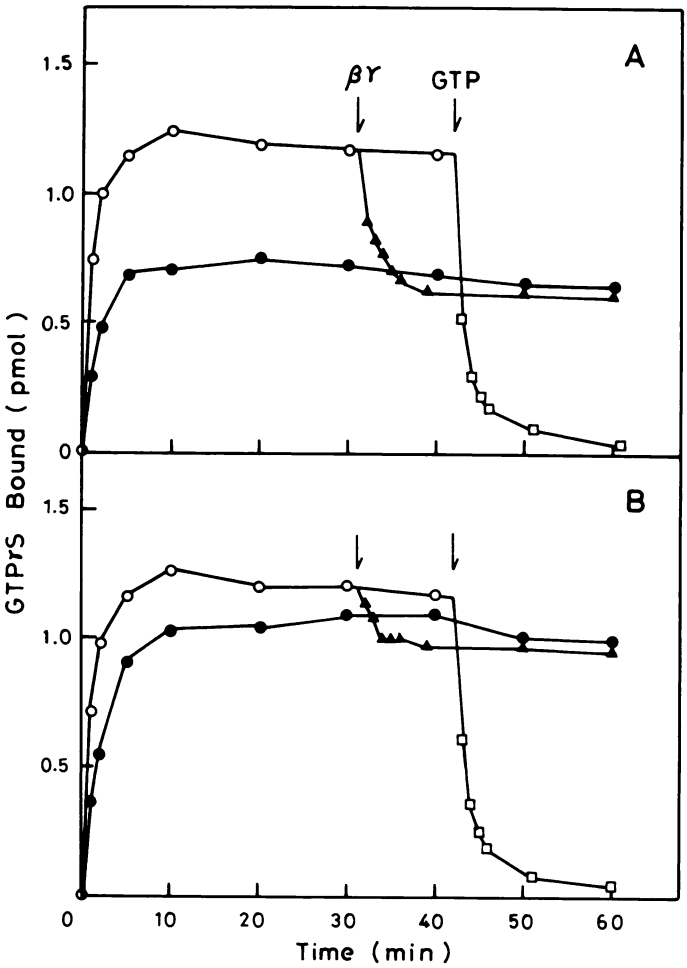

Fig. 3. GTPγS binding to NEM-treated or nontreated Goα subunit. At zero time, 4.6 μg of nontreated (A) or NEM-treated (B) Goα subunit were added to 460 μl of 20 mM Hepes (pH 8.0), 1 mM EDTA, 1 mM DTT, 0.05% Lubrol PX, and 1 μM [^{35}S]GTPγS and incubated at 30°. At the indicated times, 20-μl samples were withdrawn and assayed for [^{35}S] GTPγS binding (○). At 31 min, 180 μl of the reaction mixture was transferred to the prewarmed test tube containing 9 μg of βγ subunits (▲). At 42 min, 0.25 mM GTP was added to the reaction mixture (□). At zero time, the reaction mixture contained 2 μg of Goα and 10 μg of βγ (●).

the high affinity of GABA binding with GTP-binding proteins in NEM-treated membranes indicates that GTP-binding protein is impaired by NEM.

Among the various properties of GTP-binding proteins, ADP-ribosylation by IAP and the ability to couple to $GABA_B$ receptors were affected by NEM. When α- and βγ-subunits of GTP-binding proteins were treated with NEM separately and then reassociated with nontreated subunits, Gi and Go with NEM-treated α-subunits could not be IAP substrates and did not couple to GABA receptors. These results indicate that uncoupling by NEM is due to modification of the α-subunit of GTP-binding proteins. Previous studies demonstrated that IAP abolished receptor-mediated inhibition of adenylate cyclase or receptor-mediated stimulation of GTPγS binding but did not alter Gpp(NH)p-induced inhibition of cyclase or Mg^{2+}-stimulated GTPγS binding (11, 12). Modification of α subunit by either IAP or NEM resulted in the inability of GTP-binding protein to interact with receptor. These results suggest that the site modified by NEM is on or near the site of ADP-ribosylation

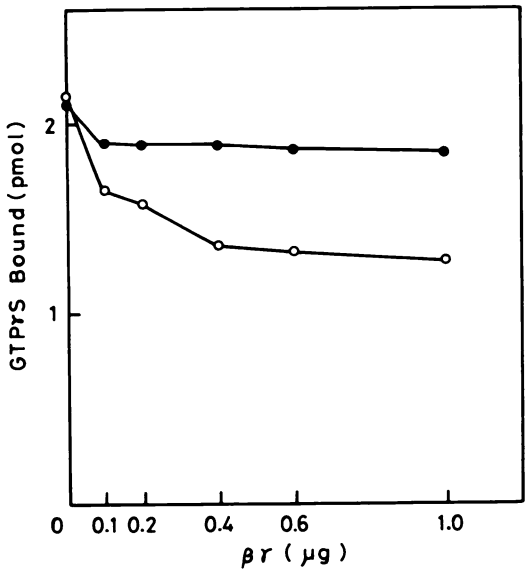

Fig. 4. Effect of $\beta\gamma$ on GTPγS binding to Goα subunit. NEM-treated (●) or nontreated (○) Goα subunit (0.2 μg) was incubated wtih the indicated amount of $\beta\gamma$ subunits in 50 μl of 20 mM Hepes (pH 8.0), 1 mM EDTA, 1 mM DTT, 0.05% Lubrol PX, and 1 μM [^{35}S]GTPγS at 30° for 15 min.

by IAP and that cysteine is most likely to be ADP-ribosylated by IAP. In the case of transducin, which is a GTP-binding protein similar to Gi or Go, the site of ADP-ribosylation by IAP appears to be near the carboxyl terminus (26). The amino acid sequence of the α-subunit of transducin reveals the existence of a cysteine near the carboxyl terminus (27–29), and the authors speculated that the cysteine residue might be the target for ADP-ribosylation (27, 29). The results also suggest that NEM causes the uncoupling of GTP-binding proteins from receptor by the same mechanism as IAP.

When Gi and Go oligomers were treated with NEM, GTPγS binding and GTPase activity were not affected. However, when isolated subunits were treated with NEM, both GTPγS binding in the absence of Mg^{2+} and GTPase activity were changed. These changes were due to the modification of α by NEM, and $\beta\gamma$ was not functionally altered. These results indicate that there is another sulfhydryl group on α and that, by the binding of $\beta\gamma$, it is protected from NEM. The modification of this sulfhydryl group by NEM caused stimulation of the GTPase activity of Goα. Furthermore, the results were NEM-treated α indicate that the interaction of α with $\beta\gamma$ is diminished by NEM treatment of α. When subunits were treated with NEM, the GTPase activity of Gi in the presence of 20 mM $MgCl_2$ was decreased. In contrast, the GTPase activity of Go in the presence of 2 mM $MgCl_2$ was increased by NEM treatment (Table 2). When the activity of Gi was measured in the presence of 2 mM $MgCl_2$, it was increased by NEM treatment. Katada *et al.* (30) have found a complicated influence of Mg^{2+} on GTPase; at a low concentration of Mg^{2+}, $\beta\gamma$ decreased the GTPase activity of α but increased the activity at a high concentration of Mg^{2+}. At a low concentration of Mg^{2+}, the GTPase activity of Goα was much higher than that of Giα (30). Since our Giα preparations contained about 10% Goα, the GTPase activity of Gi was measured at a higher concentration of Mg^{2+} to diminish the activity derived from Goα. Although these data are insufficient for a complete description, the modification of this sulfhydryl group by NEM caused an increase in the GTPase activity of Goα and appeared to interfere with the interaction between α- and $\beta\gamma$-subunits.

Acknowledgments

We thank Dr. Michio Ui for providing IAP and Dr. P. Krogsgaard-Larsen for providing isoguvacine.

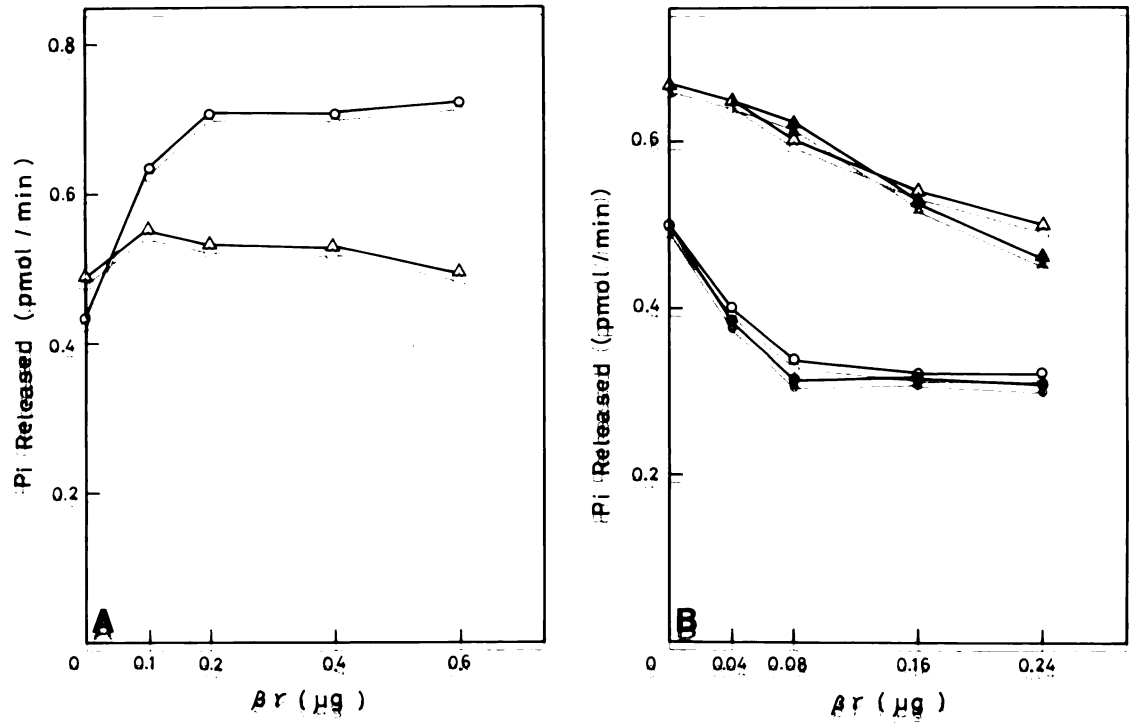

Fig. 5. Effect of $\beta\gamma$ on GTPase activity of Giα and Goα subunits. A. Nontreated (○) or NEM-treated (△) Giα subunit (0.2 μg) was incubated with the indicated amount of nontreated $\beta\gamma$ subunits in the presence of 20 mM $MgCl_2$ for 10 min at 30°. B. Nontreated (○, ●) or NEM-treated (△, ▲) Goα subunit (0.08 μg) was incubated with the indicated amount of nontreated (○, △) or NEM-treated (●, ▲) $\beta\gamma$ subunits in the presence of 2 mM $MgCl_2$.

References

1. Rodbell, M. The role of hormone receptors and GTP-regulatory proteins in membrane transduction. *Nature (Lond.)* **284**:17–22 (1980).
2. Harden, T. K., A. G. Scheer, and M. M. Smith. Differential modification of the interaction of cardiac muscarinic cholinergic and *beta*-adrenergic receptors with a guanine nucleotide binding component(s). *Mol. Pharmacol.* **21**:570–580 (1982).
3. Nukada, T., T. Haga, and A. Ichiyama. Muscarinic receptor in porcine caudate nucleus. II. Different effects of *N*-ethylmaleimide on [^{3}H]*cis*-methyldioxolane binding to heat-labile (guanyl nucleotide-sensitive) sites and heat-stable (guanyl nucleotide-insensitive) sites. *Mol. Pharmacol.* **24**:374–379 (1983).
4. Kilpatric, B. F., A. De Lean, and M. G. Caron. Dopamine receptor of the porcine anterior pituitary gland. Effect of *N*-ethylmaleimide and heat on ligand binding mimic the effect of guanine nucleotides. *Mol. Pharmacol.* **22**:298–303 (1982).
5. Sibley, D. R., and I. Creese. Regulation of ligand binding to pituitary D-2 dopaminergic receptors. Effect of divalent cations and functional group modification. *J. Biol. Chem.* **258**:4957–4965 (1983).
6. Larsen, N. E., D. Mullikin-Kilpatric, and A. J. Blume. Two different modifications of the neuroblastoma × glioma hybrid opiate receptors induced by *N*-ethylmaleimide. *Mol. Pharmacol.* **20**:255–262 (1981).
7. Yeung, S.-M. H., and R. D. Green. Agonist and antagonist affinities for inhibitory adenosine receptors are reciprocally affected by 5′-guanylylimidodiphosphate or *N*-ethymaleimide. *J. Biol. Chem.* **258**:2334–2339 (1983).
8. Jakobs, K. H., P. Lasch, M. Minuth, K. Aktories, and G. Schults. Uncoupling of α-adrenoceptor-mediated inhibition of human platelet adenylate cyclase by *N*-ethylmaleimide. *J. Biol. Chem.* **257**:2829–2833 (1982).
9. Ui, M. Islet-activating protein, pertussis toxin: a probe for functions of the inhibitory guanine nucleotide regulatory component of adenylate cyclase. *Trends Pharmacol. Sci.* **5**:277–279 (1984).
10. Kurose, H., T. Katada, T. Amano, and M. Ui. Specific uncoupling by islet-activating protein, pertussis toxin, of negative signal transduction via α-adrenergic, cholinergic, and opiate receptors in neuroblastoma × glioma hybrid cells. *J. Biol. Chem.* **258**:4870–4875 (1983).
11. Cote, T. E., E. A. Frey, and R. D. Sekura. Altered activity of the inhibitory guanyl nucleotide-binding component (Ni) induced by pertussis toxin. Uncoupling of Ni from receptor with continued coupling of Ni to the catalytic unit. *J. Biol. Chem.* **259**:8693–8698 (1984).
12. Asano, T., T. Katada, A. G. Gilman, and E. M. Ross. Activation of the inhibitory GTP-binding protein of adenylate cyclase, Gi, by β-adrenergic receptors in reconstituted phospholipid vesicles. *J. Biol. Chem.* **259**:9351–9354 (1984).
13. Okajima, F., T. Katada, and M. Ui. Coupling of the guanine nucleotide regulatory protein to chemotactic peptide receptors in neutrophil membranes and its uncoupling by islet-activating protein, pertussis toxin. A possible role of the toxin substrate in Ca^{2+}-mobilizing receptor-mediated signal transduction. *J. Biol. Chem.* **260**:6761–6768 (1985).
14. Sternweis, P. C., and J. D. Robishaw. Isolation of two proteins with high affinity for guanine nucleotides from membranes of bovine brain. *J. Biol. Chem.* **259**:13806–13813 (1984).
15. Neer, E. J., J. M. Lok, and L. G. Wolf. Purification and properties of the inhibitory guanine nucleotide regulatory unit of brain adenylate cyclase. *J. Biol. Chem.* **259**:14222–14229 (1984).
16. Wojcik, W. J., and N. H. Neff. γ-Aminobutyric acid B receptors are negatively coupled to adenylate cyclase in brain, and in the cerebellum these receptors may be associated with granule cells. *Mol. Pharmacol.* **25**:24–28 (1984).
17. Wojcik, W. J., D. Cavalla, and N. H. Neff. Co-localized adenosine A1 and γ-aminobutyric acid B ($GABA_B$) receptors of cerebellum may share a common adenylate cyclase catalytic unit. *J. Pharmacol. Exp. Ther.* **232**:62–66 (1985).
18. Hill, D. R., N. G. Bowery, and A. L. Hudson. Inhibition of $GABA_B$ receptor binding by guanyl nucleotides. *J. Neurochem.* **42**:652–657 (1984).
19. Asano, T., M. Ui, and N. Ogasawara. Prevention of the agonist binding to γ-aminobutyric acid B receptors by guanine nucleotides and islet-activating protein, pertussis toxin, in bovine cerebral cortex. Possible coupling of the toxin-sensitive GTP-binding proteins to receptors. *J. Biol. Chem.* **260**:12653–12658 (1985).
20. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**:265–275 (1951).
21. Katada, T., G. M. Bokoch, J. K. Northup, M. Ui, and A. G. Gilman. The inhibitory guanine nucleotide-binding regulatory component of adenylate cyclase. Properties and function of the purified protein. *J. Biol. Chem.* **259**:3568–3577 (1984).
22. Schaffner, W., and C. Weissmann. A rapid, sensitive, and specific method for the determination of protein in dilute solution. *Anal. Biochem.* **56**:502–514 (1973).
23. Northup, J. K., M. D. Smigel, and A. G. Gilman. The guanine nucleotide activating site of the regulatory component of adenylate cyclase. Identification by ligand binding. *J. Biol. Chem.* **257**:11416–11423 (1982).
24. Bokoch, G. M., T. Katada, J. K. Northup, M. Ui, and A. G. Gilman. Purification and properties of the inhibitory guanine nucleotide-binding regulatory component of adenylate cyclase. *J. Biol. Chem.* **259**:3560–3567 (1984).
25. Brandt, D. R., T. Asano, S. E. Pedersen, and E. M. Ross. Reconstitution of catecholamine-stimulated guanosinetriphosphatase activity. *Biochemistry* **22**:4357–4362 (1983).
26. Manning, D. R., B. A. Fraser, R. A. Kahn, and A. G. Gilman. ADP-ribosylation of transducin by islet-activating protein. Identification of asparagine as the site of ADP-ribosylation, *J. Biol. Chem.* **259**:749–756 (1984).
27. Hurley, J. B., M. I. Simon, D. B. Teplow, J. D. Robishaw, and A. G. Gilman. Homologies between signal transducing G proteins and *ras* gene products. *Science* **226**:860–862 (1984).
28. Tanabe, T., T. Nukada, Y. Nishikawa, K. Sugimoto, H. Suzuki, H. Takahashi, M. Noda, T. Haga, A. Ichiyama, K. Kangawa, N. Minamino, H. Matsuo, and S. Numa. Primary structure of the α-subunit of transducin and its relationship to *ras* proteins. *Nature (Lond.)* **315**:242–245 (1985).
29. Medynski, D. C., K. Sullivan, D. Smith, C. V. Dop, F. H. Chang, B. K.-K. Fung, P. H. Seeburg, and H. R. Bourne. Amino acid sequence of the α subunit of transducin deduced from the cDNA sequence. *Proc. Natl. Acad. Sci. USA* **82**:4311–4315 (1985).
30. Katada, T., M. Oinuma, and M. Ui. Two guanine nucleotide-binding proteins in rat brain serving as the specific substrate of islet-activating protein, pertussis toxin. Interaction of the α-subunits with $\beta\gamma$-subunits in development of their biological activities. *J. Biol. Chem.*, in press.

Send reprint requests to: Dr. Tomiko Asano, Department of Biochemistry, Institute for Developmental Research, Aichi Prefectural Colony, Kasugai, Aichi 480-03, Japan.