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Cerebral Muscarinic Acetylcholine Receptors Interact with Three Kinds of GTP-Binding Proteins in a Reconstitution System of Purified Components

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SUMMARY

A new GTP-binding protein, which serves as a substrate for pertussis toxin, was prepared from porcine brain. The new G protein was separated from other GTP-binding proteins, G_i and G_o, by an anion-exchange column chromatography. The mobility on sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the α subunit of the new G protein was between those of α subunits of G_i and G_o. Evidence that the α subunit is not a proteolytic fragment of the α subunit of G_i or G_o was provided by experiments involving partial hydrolysis of these G proteins with thermolysin and their interaction with an antibody raised against the amino terminal peptide of the α subunit of G_i. In addition, the γ subunit of the new G protein was indicated to be different from the γ subunits of G_i and G_o, because the latter were found to be phosphorylated by protein kinase C but the former was not. GTP-sensitive high affinity binding of muscarinic receptors with acetylcholine was observed when muscarinic receptors purified from porcine cerebrum were reconstituted in phospholipid vesicles with the new G protein as well as with G_i or G_o. The proportion of the high affinity sites increased with the concentrations of the G proteins, the potency of the new G protein being similar to that of G_i but a little lower than that of G_o. This GTP-sensitive high affinity binding was not observed when each G protein was pretreated with pertussis toxin and then reconstituted with muscarinic receptors. Acetylcholine accelerated the dissociation of [^{3}H]GDP from the new G protein as well as from G_i and G_o, which were reconstituted with muscarinic receptors. These results indicate that muscarinic receptors interact with at least the above three kinds of G proteins, in a pertussis toxin-sensitive manner.

Muscarinic acetylcholine receptors are known to induce several kinds of events, such as the inhibition of adenylate cyclase, activation of polyphosphoinositide phosphodiesterase, and regulation of ion channels. Data have been accumulating that indicate that these responses are mediated through the activation of G proteins by muscarinic receptors (for a review, see Ref. 1). The similarity of the structures of muscarinic receptors to those of rhodopsin and β-adrenergic receptors also supports this idea (2–5). The interaction of muscarinic receptors with G proteins has been demonstrated in reconstituted systems (6–10); brain muscarinic receptors, which were resolved from G proteins (6) or purified to apparent homogeneity (7–9), interacted with two kinds of G proteins (G_i and G_o),[2] which were purified from brain (11). The interaction of G_i and muscarinic receptors, both of which were purified from the atrium, was also reported (10). The interaction of purified components was inhibited by treatment of G_i with pertussis toxin (8, 10). The α subunits of G_i and G_o are known to be ADP-ribosylated by pertussis toxin (11, 12).

Recent studies have indicated that there are three kinds of

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[2] G_i, G_o and G_s are GTP-binding regulatory proteins isolated from porcine brain and composed of three subunits, $\alpha\beta\gamma$. G_i, G_o, and G_s are separated from each other with an anion-exchange gel chromatography and eluted from the gel in this order. G_i, G_o, and G_s are also characterized by apparent molecular size of the α subunits on SDS-PAGE and often termed as $\alpha_{41}\beta\gamma$, $\alpha_{39}\beta\gamma$, and $\alpha_{46}\beta\gamma$, respectively. Five kinds of cDNA for α subunits of G proteins except G_s and transducin have been identified and termed as $G_{i\alpha1}$ (or $G_{i1\alpha}$), $G_{i\alpha2}$ (or $G_{i2\alpha}$), $G_{i\alpha3}$ (or $G_{i3\alpha}$), $G_{o\alpha}$, and $G_{z\alpha}$ (or $G_{z\alpha}$). The α subunits of G_i, G_o, and G_s are considered to be mostly composed of $G_{i\alpha1}$, $G_{o\alpha}$, $G_{i\alpha2}$, respectively, but the possibility remains that they also include $G_{i\alpha3}$, $G_{z\alpha}$, and unidentified proteins. Two kinds of cDNA for β subunits have been identified but it is not known how they correspond to the β subunits of G_i, G_o, and G_s. cDNA for the γ subunit has not been identified, except that for transducin.

ABBREVIATIONS: G protein, GTP-binding protein; [^{3}H]QNB, [^{3}H]L-quinuclidinyl benzilate; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; GTPγS, guanosine 5′ (3-O-thio)triphosphate; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; DTT, dithiothreitol.

cDNAs that encode the α subunits of G_i ($G_{i\alpha1}$, $G_{i\alpha2}$, and $G_{i\alpha3}$) (12–18). The deduced amino acid sequences indicate that all three α subunits serve as substrates for ADP-ribosylation by pertussis toxin. One of these α subunits ($G_{i\alpha1}$) is considered to be a predominant component of the G_i preparation that was isolated from bovine brain, because partial amino acid sequences determined for the G_i preparation were found in the sequence of $G_{i\alpha1}$ but not necessarily in that of $G_{i\alpha2}$ or $G_{i\alpha3}$ (12, 13). A G_i preparation obtained by essentially the same method from rat or porcine brain was used for reconstitution with muscarinic receptors (7–9). Recently, Katada *et al.* (19) prepared a G protein from porcine brain that served as a substrate for pertussis toxin but that was different from G_i and G_o in the apparent size of the α subunit on SDS-PAGE. The α subunit of the new G protein appeared to correspond to $G_{i\alpha2}$ on comparison of the partial amino acid sequences (20). Mumby *et al.* (21) reported the isolation of an α subunit from bovine brain, which appeared to correspond to $G_{i\alpha2}$ on the basis of immunological specificity.

We also prepared a G protein from porcine brain, which was tentatively designated as G_n. In the present paper, we report that 1) the α subunit of G_n is distinct from those of G_i and G_o and it is likely to correspond to $G_{i\alpha2}$, 2) the γ subunit of G_n was also different from those of G_i or G_o in availability as substrates for phosphorylation by protein kinase C, and 3) muscarinic receptors purified from porcine brain interacted with G_n as well as G_i and G_o in a pertussis toxin-sensitive manner.

Experimental Procedures

Materials. [^{3}H]QNB (39 Ci/mmol) and [^{35}S]GTPγS (1013 Ci/mmol) were purchased from Amersham Corp. (Arlington Heights, IL); [^{14}C]NAD (0.6 Ci/mmol) and ^{14}C-methylated marker proteins from New England Nuclear (Boston, MA); [^{32}P]ATP from ICN Radiochemicals (Plainview, NY); digitonin from Wako Pure Chemical Industries and Sigma Chemical Company (St. Louis, MO); histone III S, thermolysin (EC 3.4.24.4.), and a brain extract (Folch fraction I) from Sigma; and phenyl-Sepharose from Pharmacia Fine Chemicals. Threonine-Sepharose was prepared as described (22). Pertussis toxin was kindly donated by Drs. M. Ui and M. Yajima.

Purification of muscarinic receptors and G proteins. Muscarinic receptors were purified from porcine cerebrum by means of single affinity chromatography as described previously (specific [^{3}H]QNB binding activity, 2–4 nmol/mg of protein) (23). A mixture of G_i and G_o was prepared from porcine brain (200 g of wet weight) as described by Sternweis and Robishaw (11) with a slight modification (9). G_i was separated from G_o on a column of DEAE-Toyopearl 650S (12 × 1.5 cm, 20 ml) (Fig. 1); they were eluted with a linear gradient of NaCl (0–0.25 M) in 20 mM Tris·HCl buffer (pH 8.0), 1 mM EDTA, and 1 mM DTT (TED solution) containing 0.6% Lubrol PX (total volume, 200 ml). The second peak, which showed [^{35}S]GTPγS-binding activity, had a shoulder. This shoulder had already been detected in previous studies (see Fig. 2 in Ref. 9) and had been reproducibly observed thereafter. The pooled shoulder fractions (fractions 64 to 70 in Fig. 1), obtained in two repeated experiments, were pooled and then subjected to rechromatography on the same column (Fig. 1). Fractions 64 to 70 obtained on the rechromatography were used as the G_n preparation.

Reconstitution of muscarinic receptors and G proteins. A lipid mixture was prepared as described (9). Briefly, the bovine brain extract (Folch fraction I) and porcine total lipids (1.5 mg each) were suspended in 1 ml of a solution (HEN; 20 mM Hepes-KOH buffer (pH 8.0), 1 mM EDTA, and 160 mM NaCl) containing 0.18% deoxycholate and 0.04% sodium cholate. Receptor preparations [0.2–0.4 nmol/ml [^{3}H]QNB binding sites in 500 mM phosphate buffer (pH 7.0) and 0.1% digitonin, 10–40 μl) were mixed with 0.1 mM oxotremorine in HEN and then with 100 μl of the lipid mixture (final volume, 200 μl), followed by passage through a small column of Sephadex G-50 (0.7 × 4.6 cm) preequilibrated with HEN. The void volume fraction (1–8 pmol of [^{3}H]QNB binding sites, 400 μl) was mixed with G proteins [0–200 pmol of [^{35}S]GTPγS binding sites in 40 μl of a cholate solution (TED; 0.3 or 0.8% sodium cholate and 0.1 M NaCl)] and HEN (50 μl) containing $MgCl_2$ and DTT (final concentrations, 10 and 5 mM, respectively); the medium for G proteins had been changed from a Lubrol solution to the cholate solution through the use of hydroxyapatite as described (9). The mixture of receptors and G proteins was kept for 60 min at 0°, diluted with 3 to 5 volumes of HEN, and then used for the [^{3}H]QNB binding assay as described (9) except that 5 mM DTT was added. The same preparation was used for the [^{3}H]GDP binding assay. The recoveries of [^{3}H]QNB and [^{35}S]GTPγS binding activity after reconstitution ranged from 30% to 50%.

Partial hydrolysis of G proteins. G proteins in the Lubrol solution or in the 0.8% cholate solution (100 pmol of [^{35}S]GTPγS binding sites, 50 μl) were mixed with 50 μl of TED-0.6% Lubrol PX, containing or not containing 60 mM $MgCl_2$ and 20 μM GTPγS, and then with thermolysin (0.2 mg/ml, 20 μl), followed by incubation at 30° for 0–60 min. A portion of the reaction mixture (20 μl) was subjected to SDS-PAGE analysis. In some experiments, G proteins treated with thermolysin for 60 min were applied to a DEAE-Toyopearl column (1 ml), preequilibrated with a solution containing TED, 0.6% Lubrol PX, and 10 mM $MgCl_2$, and then eluted with a linear gradient of NaCl (0 to 0.2 M) in the same solution (total volume, 20 ml). An aliquot (20 μl) of each fraction (0.4 ml) was subjected to SDS-PAGE.

ADP-ribosylation of G proteins. ADP-ribosylation of G proteins with pertussis toxin was carried out as described by Bokoch *et al.* (24) except that [^{14}C]NAD was used instead of [^{32}P]NAD. Eluates from the DEAE-Toyopearl column or G proteins in the cholate solution were diluted 20- to 50-fold with a solution containing 75 mM Tris·HCl buffer (pH 8.0), 10 mM thymidine, 2.5 mM $MgCl_2$, 1 mM EDTA, 1 mM DTT, 1 mM ATP, and 1 mg/ml L-α-dimyristoyl phosphatidylcholine (total volume, 80 μl), followed by incubation with 25 μg/ml pertussis toxin and 0.85 μM [^{14}C]NAD or 100 μM unlabeled NAD at 30° for 60 min. The ADP-ribosylated G proteins were trapped on membrane filters, after the addition of trichloroacetic acid, or subjected to SDS-PAGE followed by autoradiography using EN^{3}HANCE (New England Nuclear). In some experiments, ADP-ribosylated G proteins were used for reconstitution with muscarinic receptors or were subjected to partial hydrolysis with thermolysin.

Purification of protein kinase C. Protein kinase C was purified from porcine brain as described (22). Briefly, an extract from 60 g of porcine brain was subjected to successive column chromatographies on DEAE-cellulose (DE52), threonine-Sepharose, and phenyl-Sepharose. In the final step we detected two peaks exhibiting protein kinase activity; fractions corresponding to the first and second peaks were tentatively termed C kinases I and II, respectively. Both the C kinase I and II activities were dependent on the presence of Ca^{2+} and phospholipids, but were not affected by cyclic AMP, and were approximately 5–10 units/ml, where one unit is defined as the amount of enzyme that incorporates 1 nmol of phosphate from ATP (10 μM) into histone per minute at 30°. Each fraction was mixed with an equal volume of 20% glycerol/0.1% Tween 20 and then stored at −80°, without a decrease in activity for at least 8 months.

Phosphorylation of G proteins. G proteins in the cholate solution were diluted 3-fold with HEN, mixed with an equal volume of the lipid mixture, and then passed through a Sephadex G-50 column preequilibrated with HEN. The void volume fraction was used as the G proteins reconstituted into phospholipid vesicles. G proteins in the cholate solution or ones reconstituted into phospholipid vesicles (100 pmol of [^{35}S]GTPγS binding sites, 100 μl) were phosphorylated with an enzyme preparation (5–10 μl of the C kinase I or II) in a medium containing 1 or 10 μM [^{32}P]ATP (100–1000 cpm/pmol), 0.5 mM $CaCl_2$, 5 mM Mg-acetate, 10 μg of brain extract, and 0.2 μg of diolein (total volume, 250 μl) at 30°. After the incubation, an aliquot (20 μl) was mixed with an equal volume of a solution containing 5% SDS, followed by SDS-PAGE (acrylamide concentration, 15%) according to the method of Laemmli

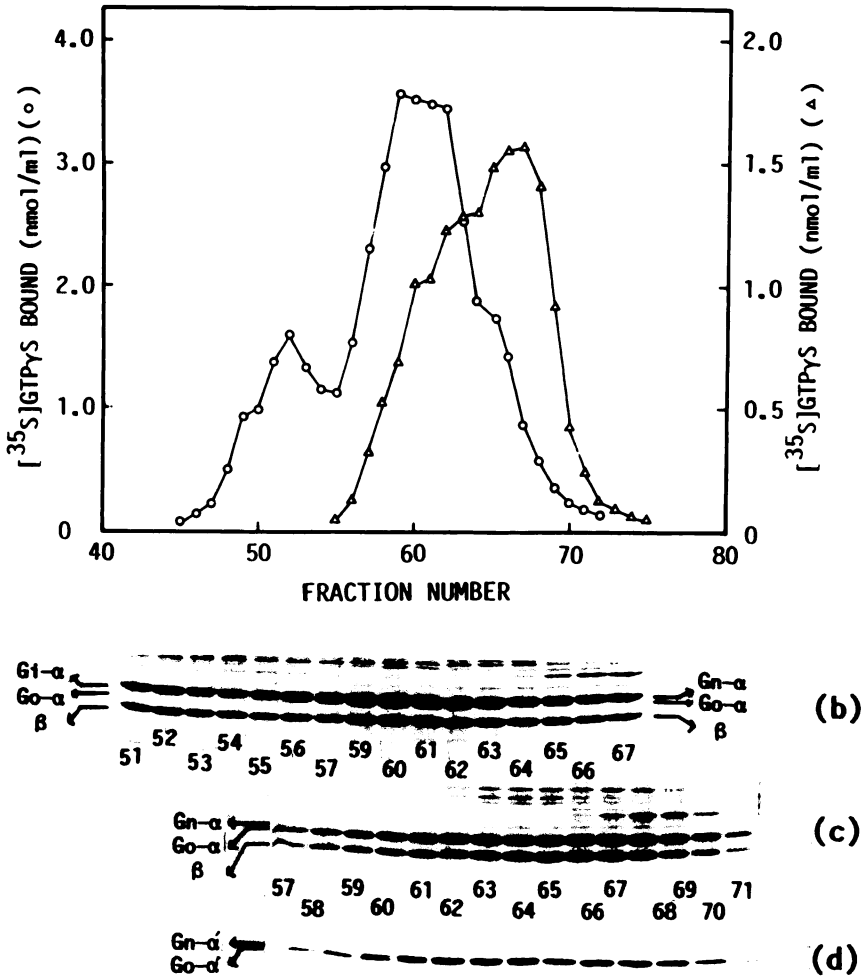

Fig. 1. Preparation of G_n on DEAE-Toyopearl. The experimental details are given under Experimental Procedures. Fractions 64 to 70 obtained on the first chromatography (○), obtained in two experiments carried out independently, were pooled and then subjected to rechromatography. Fractions 64 to 70 obtained on the rechromatography (△) were used as the G_n preparation. A portion (5 μl) of each fraction was used for the [^{35}S]GTPγS binding assay and another portion (20 μl) for SDS-PAGE (acrylamide concentration, 12%) followed by silver staining (b, first chromatography; c, rechromatography). A portion of each fraction obtained on the rechromatography was subjected to [^{14}C]ADP-ribosylation with pertussis toxin, followed by SDS-PAGE and then autoradiography (d).

(25). Alternatively, 250 μl of 50% trichloroacetic acid was added to the reaction mixture and then the suspension was centrifuged. The precipitate was washed with 5% trichloroacetic acid and then with ethyl ether and was finally suspended in a solution containing 2.5% SDS. The final suspension was subjected to SDS-PAGE. The α and γ subunit bands were cut out and the radioactivity in them was eluted by electrophoresis at 100 V for 2 hr in a medium containing 2.5 mM Tris, 19.2 mM glycine, and 0.01% SDS. The eluate was hydrolyzed with 6 M HCl under an atmosphere of N_2 at 110° for 2 hr, and the hydrolysate was lyophilized, mixed with cold phosphoserine, phosphothreonine, and phosphotyrosine, and then subjected to high voltage paper electrophoresis (2.5 kV for 30 min) in pyridine/acetic acid/H_2O (1:10:89, v/v, pH 3.5) in order to determine phosphorylated amino acids. The amount of phosphate incorporated into each subunit was estimated by Cerenkov counting of the respective band cut out from the gel after SDS-PAGE, followed by correction as to the counting efficiency.

Immunoassay. The 32-amino terminal peptide of the α subunit of bovine brain G_i was synthesized with an automatic peptide-synthesizer by Hoechst Japan Ltd. (Kawagoe, Japan); its sequence is CTVSAED-KAAAERSKMIDKNLREDGEKAAREV (13). The peptide was conjugated with keyhole limpet hemocyanin and then injected into rabbits. Immunoreactivity was detected using donkey anti-rabbit IgG conjugated with biotin and horseradish peroxidase-streptavidin (Amersham).

Other methods. Binding assays for [^{3}H]QNB or [^{35}S]GTPγS and analysis of binding data with a computer were carried out as described in the previous paper (9).

Results

Preparation of G_n. G_i and G_o were separated from each other on a column of DEAE-Toyopearl as described previously (9); the first and second peaks, which exhibited [^{35}S]GTPγS binding activity, corresponded to G_i and G_o, respectively (Fig. 1). The second peak had a shoulder, which appeared after the major portion of G_o had been eluted. The fractions forming the shoulder were collected and subjected to rechromatography on the same column, which resulted in the elution of a peak of [^{35}S]GTPγS-binding activity in fractions distinct from those in which G_o had been eluted. We assumed that a new G protein was eluted behind G_o and designated this G protein as G_n.

G_n was composed of αβγ subunits like G_i and G_o, and the mobility on SDS-PAGE of $G_{n\text{-}\alpha}$ was between those of $G_{o\text{-}\alpha}$ and $G_{i\text{-}\alpha}$ (Fig. 2). G_n, as well as G_i and G_o, was ADP-ribosylated with pertussis toxin and the ratio of [^{14}C]ADP-ribose incorporated and [^{35}S]GTPγS bound was near to 1 (Table 1). The differences in the mobility of the three kinds of α subunits were observed a little more clearly with [^{14}C]ADP-ribosylated samples (compare Fig. 1, b and c with d); the transition from G_o to G_n occurred in fractions 60 and 61 in Fig. 1d.

Evidence that $G_{n\text{-}\alpha}$ is not a proteolytic fragment of $G_{i\text{-}\alpha}$ or $G_{o\text{-}\alpha}$. When the G proteins were treated with thermo-

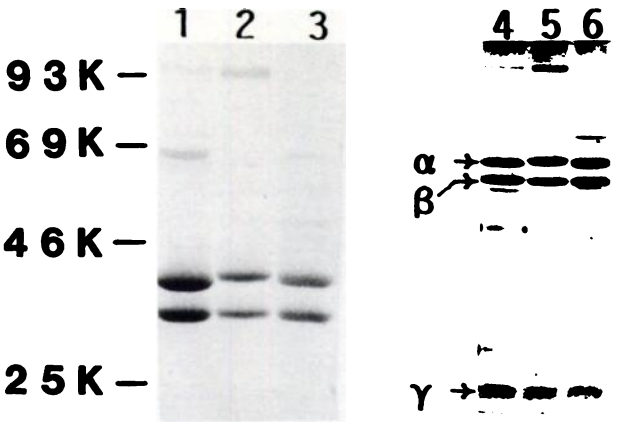

Fig. 2. SDS-PAGE of G_o, G_i, and G_n. Twenty picomoles of [^{35}S]GTPγS binding sites for each of the G_o (1 and 4), G_i (2 and 5), and G_n (3 and 6) preparations was subjected to SDS-PAGE [acrylamide, 12% (1–3) or 15% (4–6)] and then the gel was stained with Coomassie brilliant blue (1–3) or silver (4–6).

TABLE 1

[^{35}S]GTPγS binding activity and [^{14}C]ADP-ribosylation of the three G proteins, G_o, G_i, and G_n

Protein concentrations were determined as described by Schaffner and Weismann (32). [^{35}S]GTPγS binding and [^{14}C]ADP-ribosylation with pertussis toxin were carried out as described under Experimental Procedures.

	Protein concentration (a)	[^{35}S]GTPγS binding (b)	[^{14}C]ADP-ribosylation (c)	c/b	b/a
	mg/ml	*nmol/ml*	*nmol/ml*		*nmol/mg*
G_o	1.20	12.7	11.0	0.87	10.6
G_i	1.28	10.4	11.3	1.09	8.2
G_n	1.40	11.0	10.3	0.94	7.9

lysin in the presence of GTPγS, the mobilities on SDS-PAGE of the α subunits of the three G proteins increased and the apparent molecular sizes of the α subunits were estimated to decrease by 2–3 kDa (Fig. 3). The order of mobility on SDS-PAGE was $G_{o\text{-}\alpha'} > G_{n\text{-}\alpha'} = G_{i\text{-}\alpha'} > G_{o\text{-}\alpha} > G_{n\text{-}\alpha} > G_{i\text{-}\alpha}$, where $G_{o\text{-}\alpha'}$ $G_{n\text{-}\alpha}$, , and $G_{i\text{-}\alpha'}$ are the hydrolytic products of $G_{o\text{-}\alpha}$, $G_{n\text{-}\alpha}$, and $G_{i\text{-}\alpha}$, respectively.

The amino terminal peptides are considered to be cleaved off from the α subunits because the antibody raised against the amino terminal peptide of $G_{i\text{-}\alpha}$ reacted with G_i, G_n, and G_o, in that order of potency, but much more weakly with the hydrolyzates with thermolysin (data not shown). On the other hand, when the G proteins were treated with pertussis toxin and then thermolysin, the [^{14}C]ADP-ribosyl moiety, which is assumed to bind to the cysteine residue four removed from the carboxy terminus (12), remained bound to the $G_{i\text{-}\alpha'}$, $G_{o\text{-}\alpha'}$, and $G_{n\text{-}\alpha'}$, which indicates that the carboxy terminal peptides were not cleaved off by thermolysin.

Fig. 4 shows the elution patterns, from a DEAE-Toyopearl column, of the hydrolysates with thermolysin of G_i, G_o, and G_n. $G_{i\text{-}\alpha'}$, $G_{o\text{-}\alpha'}$, and $G_{n\text{-}\alpha'}$ were eluted from the column in that order, which is the same as the order of elution of the original G proteins. The β subunits were eluted in the flow-through fractions, without binding to the gel. When the G proteins were treated with pertussis toxin and then thermolysin, $G_{i\text{-}\alpha'}$, $G_{o\text{-}\alpha'}$, and $G_{n\text{-}\alpha'}$, to which the [^{14}C]ADP-ribosyl moiety was bound, were also eluted, in that order, from the same column (data not shown).

These results indicate that the different affinities of G_i, G_o, and G_n for DEAE-Toyopearl gel are due to the different affinities of their α subunits and that the differences reside in the regions between 2 and 3 kDa downstream of the amino terminals and the carboxy terminals of the α subunits. These results rule out the possibility that $G_{n\text{-}\alpha}$ is a proteolytic fragment of $G_{i\text{-}\alpha}$ or $G_{o\text{-}\alpha}$ devoid of the amino terminal or carboxy terminal peptide. Three amino acid residues in the carboxy terminals of $G_{i\text{-}\alpha}$ and $G_{o\text{-}\alpha}$ are neutral and are not likely to be involved in the interaction with the gel. If $G_{n\text{-}\alpha}$ is assumed to be a proteolytic fragment of $G_{i\text{-}\alpha}$ (or $G_{o\text{-}\alpha}$) devoid of the amino terminal peptide, the amino terminal peptide should also be absent from $G_{i\text{-}\alpha'}$ (or $G_{o\text{-}\alpha'}$) because $G_{n\text{-}\alpha}$ reacted with the antibody more than $G_{i\text{-}\alpha'}$ (or $G_{o\text{-}\alpha'}$) and the mobility on SDS-PAGE of $G_{i\text{-}\alpha'}$ (or $G_{o\text{-}\alpha'}$) was greater than that of $G_{n\text{-}\alpha}$. Therefore, the separation of $G_{n\text{-}\alpha'}$ from $G_{i\text{-}\alpha'}$ (or $G_{o\text{-}\alpha'}$) on the DEAE-Toyopearl column could not be explained by the presence or absence of the hypothetical amino terminal peptide.

Phosphorylation of G proteins by protein kinase C. The α subunits of G_o, G_i, and G_n were phosphorylated when the G proteins were reconstituted in phospholipid vesicles and then treated with either C kinase I or II but were not phosphorylated when soluble G proteins were treated with C kinase I or II without being reconstituted into phospholipid vesicles (Fig. 5). The β subunits of G_i, G_o, and G_n were also phosphorylated when the G proteins were reconstituted in phospholipid vesicles, although the degree of phosphorylation was lower for the β subunits than for the α subunits. On the other hand, the

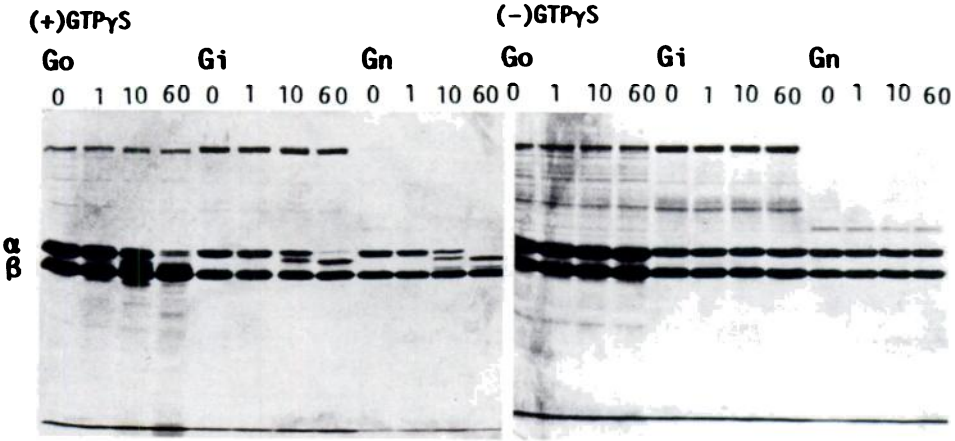

Fig. 3. Partial hydrolysis of the G proteins with thermolysin. The incubation with thermolysin was carried out at 30° for 0, 1, 10, or 60 min, as described under Experimental Procedures. These experiments were carried out in the presence or absence of 3.3 μM GTPγS and 10 mM $MgCl_2$.

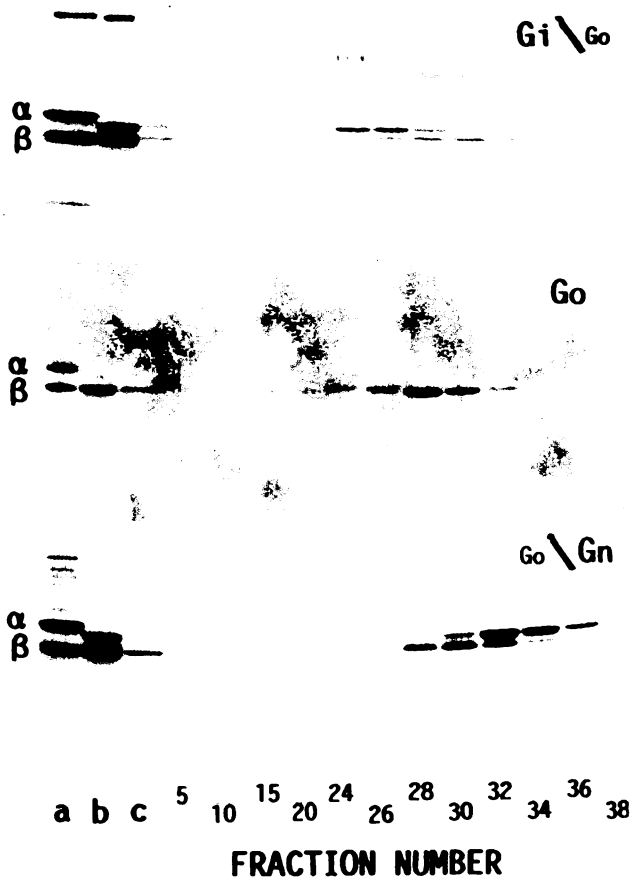

Fig. 4. DEAE-Toyopearl chromatography of the thermolysin hydrolysates of the α subunits of G_i, G_o, and G_n. A mixture of G_i and G_o (7:3), G_o, and a mixture of G_n and G_o (8:2) (approximately 120 pmol) were treated with thermolysin for 60 min and then applied to a DEAE-Toyopearl column (1 ml), followed by elution with a NaCl gradient. A portion (20 μl) of each fraction (0.4 ml) was subjected to SDS-PAGE, followed by silver staining. a, b, and c, samples before and after the thermolysin treatment and of the flow-through fraction, respectively.

γ subunits of G_i or G_o were phosphorylated regardless of whether the G proteins were reconstituted in phospholipids or were soluble. The γ subunits were phosphorylated by C kinase II better than by C kinase I, especially when the G proteins were soluble, although there was no appreciable difference between the two kinases as to the phosphorylation of the α subunits. The phosphorylation of γ subunits was greater in the presence of GTPγS than in its absence. An interesting finding was that the γ subunit of G_n was barely phosphorylated under the conditions in which the α subunits of G_o, G_i, and G_n, and the γ subunits of G_i and G_o were phosphorylated (Fig. 5). The molar amounts of incorporated phosphates were estimated to be 0.24, 0.19, and 0.02 for $G_{o\text{-}\gamma}$, $G_{i\text{-}\gamma}$, and $G_{n\text{-}\gamma}$, respectively, when G protein preparations reconstituted in phospholipids were incubated with 10 μM [^{32}P]ATP and C kinase I (80 milliunits) for 60 min at 30°. This is not likely to be due to the absence of $G_{n\text{-}\gamma}$, because a band with the same mobility as that of $G_{o\text{-}\gamma}$ or $G_{i\text{-}\gamma}$ was detected for the G_n preparation on silver staining (Fig. 2). This suggests that the γ subunit of G_n is different from those of G_o and G_i. The phosphorylated amino acid residue was serine for Go-γ and Gi-γ, as well as for all three α subunits.

Reconstitution of G proteins with muscarinic receptors. Muscarinic receptors were reconstituted with different concentrations of G_i, G_o, and G_n, and then the binding with [^{3}H]QNB in the presence of different concentrations of acetylcholine was examined (Fig. 6). Displacement curves with acetylcholine in the absence of GTP shifted leftwards with increasing concentrations of G_n, as well as of G_i or G_o, and those in the presence of GTP were essentially the same, irrespective of the concentrations of the G proteins. Thus, the higher the G concentrations, the greater the effect of GTP. These displacement curves could be explained by assuming two sites with the same affinity for [^{3}H]QNB but different affinities for acetylcholine. The proportion of the sites with high affinity for acetylcholine increased with the concentrations of the G proteins (Fig. 7). The potency of G_o was a little higher than those of G_i and G_n; the concentrations giving the half-maximal effects were 0.35, 0.49, and 0.58 nM for G_o, G_i, and G_n, respectively. The G_n preparation used here was contaminated by G_o, so there was a possibility that only G_o contributed to the formation of the binding sites with high affinity for acetylcholine. This possibility, however, does not seem to be the case, as discussed below. The proportion of G_o in this preparation was estimated by several methods, such as analyses of DEAE-Toyopearl chromatograms and SDS-PAGE patterns before and after treatment with thermolysin and/or pertussis toxin, to be around 15%. On the other hand, the proportion of the high affinity sites formed on reconstitution of receptors with the G_n preparation was much greater than that estimated on the assumptions that the proportion of G_o is 20% and that only G_o interacts with the receptors; the estimated values are shown by a *dotted line* in Fig. 7. In addition, the formation of high affinity sites was also observed for the G_n preparation obtained on DEAE-Toyopearl chromatography, repeated three times, which was apparently homogeneous. Thus, we conclude that G_n, as well as G_i and G_o, interacts with muscarinic receptors.

When a G protein, any one of G_i, G_o, and G_n, was pretreated with pertussis toxin and then reconstituted with muscarinic receptors, the high affinity binding with acetylcholine that was sensitive to GTP was greatly reduced (Fig. 8).

Another line of evidence for the interaction between muscarinic receptors and G_n was the finding that acetylcholine accelerated the dissociation of [^{3}H]GDP from G_n, as well as from G_i or G_o, reconstituted with muscarinic receptors (Fig. 9).

Discussion

The α subunit of G_n is different from those of G_i and G_o in the apparent size on SDS-PAGE. The possibility that the former is a proteolytic fragment of the latter and is devoid of the amino terminal or carboxy terminal peptide of the latter was excluded. However, there remain the possibilities that the former is a posttranslationally modified form of the latter and that both arise from the same gene but from different mRNAs, owing to alternative splicing of internal exons, and are different from each other as to some peptides inside their molecules. An alternative assumption would be that the α subunit of G_n corresponds to $G_{i\alpha2}$ or $G_{i\alpha3}$. The net charge of $G_{i\alpha1}$ was estimated to be −1 (13), and those of $G_{i\alpha2}$ and $G_{i\alpha3}$ to be −4 (17), on the assumption that lysine, arginine, and histidine residues each carry a positive charge and glutamic and aspartic acid residues each a negative charge. This is consistent with the above assumption because the α subunit of G_i ($G_{i\alpha1}$) was eluted ahead of $G_{n\text{-}\alpha}$ from the DEAE-Toyopearl column (anion-exchange gel). In addition, preliminary analysis of the amino terminal

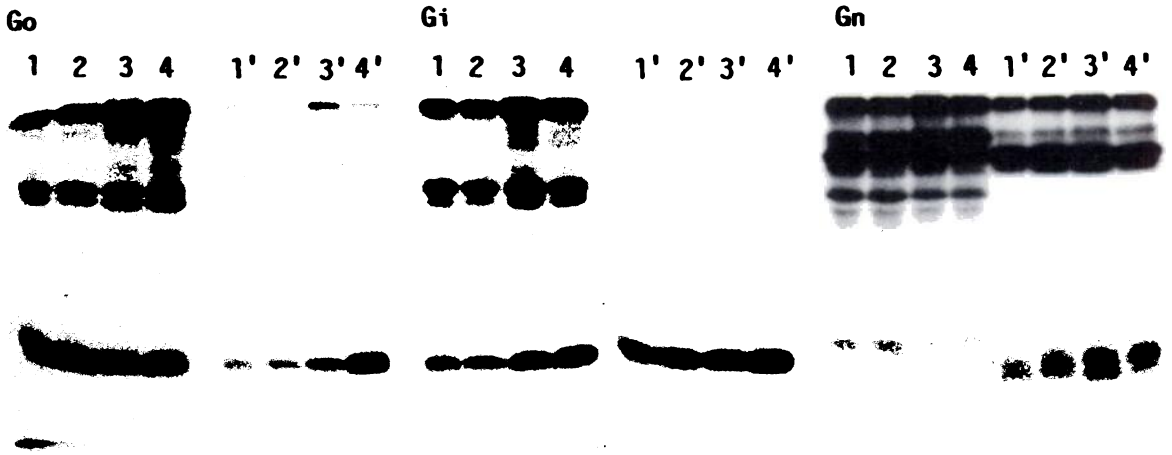

Fig. 5. Phosphorylation of the G proteins by protein kinase C. The experimental conditions are given under Experimental Procedures. Some G protein preparations were reconstituted in phospholipid vesicles and then phosphorylated with C kinase I (1 and 2) or C kinase II (3 and 4), and other preparations in the cholate solution were subjected to phosphorylation with C kinase I (1′ and 2′) or C kinase II (3′ and 4′) without reconstitution in phospholipid vesicles. Phosphorylation was carried out in the presence (2, 4, 2′, and 4′) or absence (1, 3, 1′, and 3′) of GTPγS. After reaction at 30° for 2 hr, the phosphorylated proteins were precipitated with trichloroacetic acid, and then the precipitates were washed with ether and suspended in a SDS solution. An aliquot of each suspension was subjected to SDS-PAGE (acrylamide concentration, 15%), followed by autoradiography. The bands at the *top of the gel* are considered to represent proteins that became irreversibly insoluble on the treatment with trichloroacetic acid, because these bands were not observed when an aliquot of the reaction mixture was directly subjected to SDS-PAGE.

sequences of the thermolysin hydrolysates of $G_{n\text{-}\alpha}$ indicated that the major sequence was LREDGEKAA, which is present in the sequences of $G_{i\alpha 2}$ and $G_{i\alpha 3}$ as well as that of $G_{i\alpha 1}$ (from residue 23 to 31). This assignment is not definite because the action of thermolysin was not absolutely specific and the separation of the peptides was not complete but it is consistent with the decrease in molecular mass of 2–3 kDa on treatment with thermolysin and the specificity of thermolysin, which preferentially cleaves the NH_2 side of hydrophobic residues.

During the course of the present study, Katada *et al.* (19) reported the preparation of a new G protein, which was designated as $\alpha_{40}\beta\gamma$, from porcine brain. They also used an anion-exchange gel for the separation of the $\alpha_{40}\beta\gamma$ from G_i or G_o, which were designated as $\alpha_{40}\beta\gamma$ and $\alpha_{39}\beta\gamma$ respectively, and the order of elution was G_i, G_o, and $\alpha_{40}\beta\gamma$. In addition, Mumby *et al.* (21) showed that the elution order of α subunits from an anion-exchange gel was α_{41}, α_{39}, and α_{40}. The behaviors of $\alpha_{40}\beta\gamma$ and α_{40} on anion-exchange gel are very similar to those of G_n and $G_{n\alpha}$, although different kinds of gels were used by the three groups. In addition, they presented evidence that the α_{40} subunit corresponds to $G_{i\alpha 2}$ (20, 21). Thus, it is likely that the α subunit of G_n corresponds to α_{40} and, therefore, to $G_{i\alpha 2}$.

The γ subunit of G_n was not phosphorylated by protein kinase C, although those of G_i and G_o were. This suggests that the γ subunit of G_n is also different from those of G_i and G_o, in addition to the difference in the α subunits. Several groups reported the presence of a G protein with an α subunit of an apparent size of 40 kDa in different tissues (19, 26–28). It remains to be determined whether the γ subunits of these G proteins are phosphorylated by protein kinase C. It would be interesting to determine whether there are subspecies of the γ subunits and whether the combination of the α and γ subunits is rigid or convertible.

Katada *et al.* (29) reported that solubilized $G_{i\text{-}\alpha}$ was phosphorylated by protein kinase C in the absence of $\beta\gamma$ subunits but not in the presence of the $\beta\gamma$ subunits. We also did not detect the phosphorylation of the α subunits of solubilized G proteins ($\alpha\beta\gamma$) but found that the α subunits of G_o, G_i, and G_n could be phosphorylated when the G proteins were reconstituted in phospholipid vesicles, regardless of the presence or absence of GTPγS. It remains to be determined whether the same or different residues are phosphorylated when the α subunits exist alone or with the $\beta\gamma$ subunits in phospholipid vesicles or membranes. The γ subunits of G_i and G_o were phosphorylated by protein kinase C, regardless of whether the G proteins were in the soluble form or reconstituted in phospholipid vesicles. The phosphorylation of the γ subunits appeared to vary with the species of G proteins and protein kinase C and to be affected by the presence of GTPγS, especially when the G proteins were in the soluble form. The implications of these findings and their physiological significance remain to be determined.

G_n, as well as G_i and G_o, was shown to interact with muscarinic receptors, suggesting that muscarinic receptors are capable of activating at least three distinct G proteins. The affinities of muscarinic receptors for the above three G proteins were found to be similar, although the affinity for G_o was a little higher than those for G_i and G_n. Because muscarinic receptors purified from the cerebrum may be mixtures of four different receptor subtypes (5), there is a possibility that each subtype has a high affinity for one species of G protein but that a mixture of the four subtypes has similar affinities for all the G proteins. This possibility, however, does not seem to be the case because the proportion of the high affinity sites increased to 80% with an increase in the concentration of a single species of G protein (Fig. 7) and the proportion did not increase further on the addition of both G_i and G_o, as compared with the increase when only one of the two was added (9). In addition, muscarinic receptors purified from the atrium, which are considered to be mostly composed of a single receptor subtype (mAChR II or m2) (2–5, 30), also interacted with the above three G proteins with similar affinities.[3] Thus, the interaction between purified receptors and purified G proteins does not seem to be restricted

[3] T. Ikegaya, T. Nishiyama, K. Haga, and T. Haga, unpublished results.

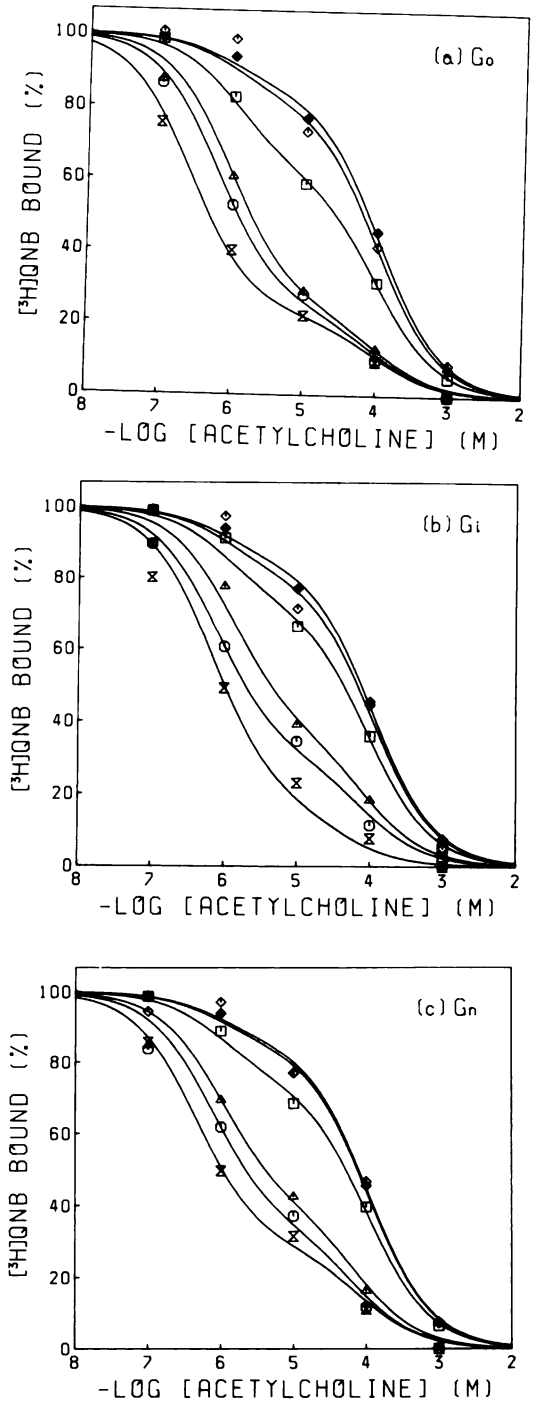

Fig. 6. Displacement by acetylcholine of [^{3}H]QNB binding to muscarinic receptors reconstituted with different concentrations of G_o, G_i, and G_n. Reconstitution of the receptors and G proteins was performed as described under Experimental Procedures. The volume and [^{3}H]QNB concentration of the reaction mixture were 0.2 ml and 1.1 nM, respectively. The concentration of muscarinic receptors in the reaction mixture was 49 pM, as determined from the [^{3}H]QNB binding activity. The highest concentrations of G_o, G_i, and G_n (⧖) in the reaction mixtures were 12.2, 10.0, and 10.6 nM, respectively, as determined from the [^{35}S]GTPγS-binding activity. The concentrations of the G proteins were as follows; 0 (♦), 1/100 (◇), 1/20 (□), 1/10 (△), and 1/5 (○) of the above highest concentrations. Guanine nucleotides were not added for these experiments. When 0.1 mM GTP was present in the reaction mixture, the displacement curves were essentially the same as those in the absence of G proteins (♦), irrespective of the concentrations of the G proteins (cf. Fig. 8). The displacement curves fitted the two-site model and the proportions of the high affinity sites are shown in Fig. 7. The concentrations of acetylcholine giving the half-maximal effects on the [^{3}H]QNB binding to the high and low affinity sites, which were estimated by curve fitting, ranged between 0.27 and 1.15 μM and between 71 and 126 μM, respectively. No systematic differences in the two values were found between the G protein species. The values for the high affinity sites showed a tendency to decrease with increasing concentrations of the G proteins.

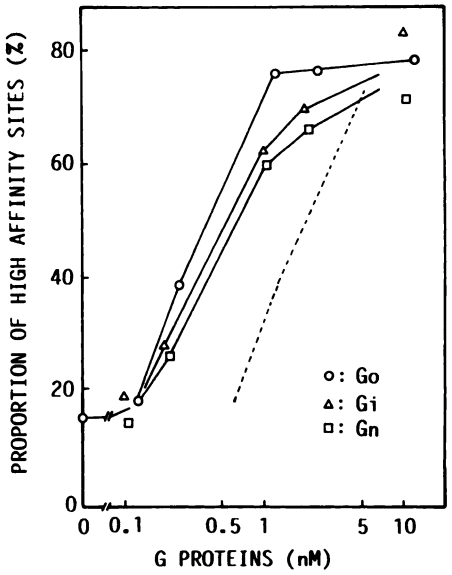

Fig. 7. Effects of the G protein concentrations on the proportion of high affinity sites. The proportions of the high affinity sites were determined by analysis of the data in Fig. 6. The *dotted line* indicates the hypothetical values calculated on the assumptions that the G_n preparation contains 80% of G_n and 20% of G_o and that only G_o interacts with muscarinic receptors; the discrepancy between the *dotted line* and the *solid line* for G_n indicates that the assumption is not valid.

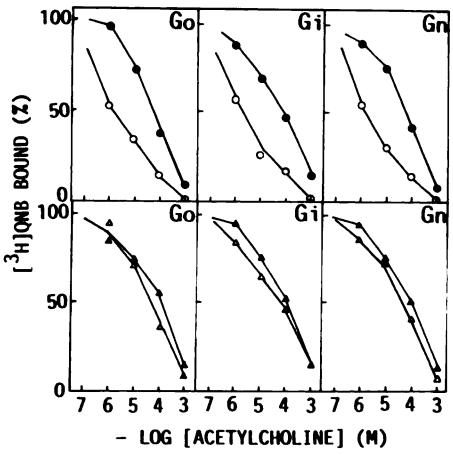

Fig. 8. Displacement by acetylcholine, in the presence (*closed symbols*) or absence (*open symbols*) of 0.1 mM GTP, of [^{3}H]QNB binding to muscarinic receptors reconstituted with G proteins treated (*triangles*) or not treated (*circles*) with pertussis toxin. The G proteins were incubated with or without pertussis toxin at 30° for 1 hr and then reconstituted with muscarinic receptors as described under Experimental Procedures.

to a specific combination of one receptor subtype and one G protein species. These results suggest that, if a single receptor subtype interacts with a single kind of G protein *in situ*, there must be some mechanism(s) or contribution by other component(s) that only allows the specific interaction.

It has been reported that the activation of polyphosphoinositide breakdown through muscarinic receptors is not inhibited

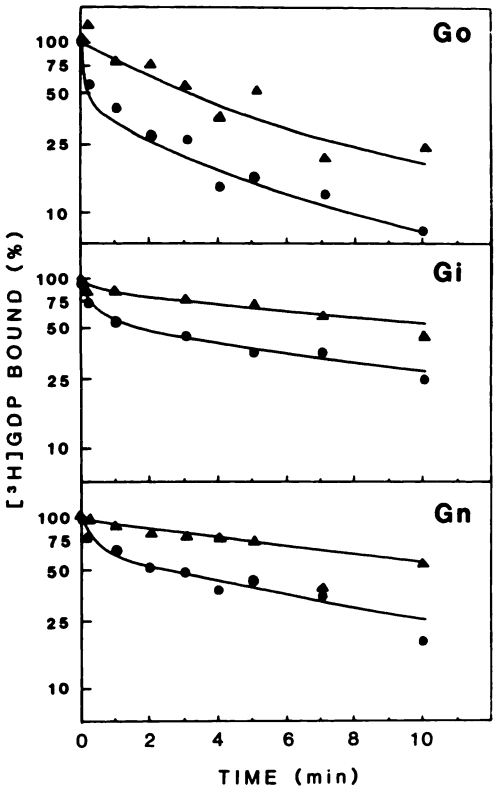

Fig. 9. Effect of acetylcholine on the release of [^{3}H]GDP from G_o, G_i, or G_n, which were reconstituted with muscarinic receptors. Reconstituted vesicles, which were prepared as described under Experimental Procedures, were incubated with 22 nM [^{3}H]GDP in the presence of 1 mM acetylcholine (●) or 10 μM atropine (▲) at 20° and, after incubation for 30 min, 0.1 mM GTP was added. After further incubation for the times indicated on the *abscissa*, aliquots were taken, and the bound form of [^{3}H]GDP was trapped on glass fiber filters and counted. The actual values for 100% were in the range of 0.4–0.6 pmol and the amount of muscarinic receptors was 30 fmol/assay.

by the treatment of cells with pertussis toxin under conditions in which other receptor-mediated processes are inhibited (see review in Ref. 31). These results suggested that muscarinic receptors activate G proteins that are not affected by pertussis toxin. G_o and G_i are known to be ADP-ribosylated by pertussis toxin, and the interaction of G_i and muscarinic receptors has been shown to be inhibited by pertussis toxin (8, 10). In the present study, the interaction of muscarinic receptors with G_o and G_n, as well as with G_i, was shown to be inhibited by pertussis toxin. So the question as to which kind of G proteins mediates the pertussis toxin-insensitive responses remains unsolved.

In summary, the present results showed that there is a novel G protein (G_n) in the brain and that muscarinic receptors are capable of interacting with at least three G proteins, G_i, G_o, and G_n, in a pertussis toxin-sensitive manner.

Acknowledgments

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References

1. Nathanson, N. M. Molecular properties of the muscarinic acetylcholine receptor. *Annu. Rev. Neurosci.* **10**:195–236 (1987).
2. Kubo, T., K. Fukuda, A. Mikami, A. Maeda, H. Takahashi, M. Mishina, T. Haga, K. Haga, A. Ichiyama, K. Kangawa, M. Kojima, H. Matsuo, and S. Numa. Cloning, sequencing and expression of complementary DNA encoding the muscarinic acetylcholine receptor. *Nature (Lond.)* **323**:411–416 (1986).
3. Kubo, T., A. Maeda, K. Sugimoto, I. Akiba, A. Mikami, H. Takahashi, T. Haga, K. Haga, A. Ichiyama, K. Kangawa, H. Matsuo, T. Hirose, and S. Numa. Primary structure of porcine cardiac muscarinic acetylcholine receptor deduced from the cDNA sequence. *FEBS Lett.* **209**:367–372 (1986).
4. Peralta, E. G., J. W. Winslow, G. L. Peterson, D. H. Smith, A. Ashkenazi, J. Ramachandran, M. I. Schimerlik, and D. J. Capon. Primary structure and biochemical properties of an M_2 muscarinic receptor. *Science (Wash. D. C.)* **236**:600–605 (1987).
5. Bonner, T. I., N. J. Buckley, A. C. Young, and M. R. Brann. Identification of a family of muscarinic acetylcholine receptor genes. *Science (Wash. D. C.)* **237**:527–532 (1987).
6. Florio, V. A., and P. C. Sternweis. Reconstitution of resolved muscarinic cholinergic receptors with purified GTP-binding proteins. *J. Biol. Chem.* **260**:3477–3483 (1985).
7. Haga, K., T. Haga, A. Ichiyama, T. Katada, H. Kurose, and M. Ui. Functional reconstitution of purified muscarinic receptors and inhibitory guanine nucleotide regulatory protein. *Nature (Lond.)* **316**:731–733 (1985).
8. Kurose, H., T. Katada, T. Haga, K. Haga, A. Ichiyama, and M. Ui. Functional interaction of purified muscarinic receptors with purified inhibitory guanine nucleotide regulatory proteins reconstituted in phospholipid vesicles. *J. Biol. Chem.* **261**:6423–6428 (1986).
9. Haga, K., T. Haga, and A. Ichiyama. Reconstitution of the muscarinic acetylcholine receptor: guanine nucleotide-sensitive high affinity binding of agonists to purified muscarinic receptors reconstituted with GTP-binding proteins (G_i and G_o). *J. Biol. Chem.* **261**:10133–10140 (1986).
10. Tota, M. R., K. R. Kahler, and M. I. Schimerlik. Reconstitution of the purified porcine atrial muscarinic acetylcholine receptor with purified porcine atrial inhibitory guanine nucleotide binding protein. *Biochemistry* **26**:8175–8182 (1987).
11. 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).
12. Gilman, A. G. G proteins. *Annu. Rev. Biochem.* **56**:615–649 (1987).
13. Nukada, T., T. Tanabe, H. Takahashi, M. Noda, K. Haga, T. Haga, A. Ichiyama, K. Kangawa, M. Hiranaga, H. Matsuo, and S. Numa. Primary structure of the α-subunit of bovine adenylate cyclase-inhibiting G-protein deduced from the cDNA sequence. *FEBS Lett.* **197**:305–310 (1986).
14. Itoh, H., T. Kozasa, S. Nagata, S., Nakamura, T. Katada, M. Ui, S. Iwai, E. Ohtsuka, H. Kawasaki, K. Suzuki, and Y. Kajiro. Molecular cloning and sequence determination of cDNAs for α subunits of the guanine nucleotide-binding proteins G_s, G_i, and G_o from rat brain. *Proc. Natl. Acad. Sci. USA* **83**:3776–3780 (1986).
15. Van Meurs, K. P., C. W. Angus, S. Lavu, H.-F. Kung, S. K. Czarnecki, J. Moss, and M. Vaughan. Deduced amino acid sequence of bovine retinal G_{as}: similarities to other guanine nucleotide-binding proteins. *Proc. Natl. Acad. Sci. USA* **84**:3107–3111 (1987).
16. Didsbury, J. R., and R. Snyderman. Molecular cloning of a new human G protein: evidence for two $G_{i\alpha}$-like protein families. *FEBS Lett.* **219**:259–263 (1987).
17. Suki, W. N., J. Abramovitz, R. Mattera, J. Codina, and L. Birnbaumer. The human genome encodes at least three nonallellic G proteins with αi-type subunits. *FEBS Lett.* **220**:187–192 (1987).
18. Jones, D. T., and R. R. Reed. Molecular cloning of five GTP-binding protein cDNA species from rat olfactory neuroepithelium. *J. Biol. Chem.* **262**:14241–14249 (1987).
19. Katada, T., M. Oinuma, K. Kusakabe, and M. Ui. A new GTP-binding protein in brain tissues serving as the specific substrate of islet-activating protein, pertussis toxin. *FEBS Lett.* **213**:353–358 (1987).
20. Itoh, H., T. Katada, M. Ui, H. Kawasaki, K. Suzuki, and Y. Kaziro. Identification of three pertussis toxin substrates (41, 40 and 39 kDa proteins) in mammalian brain. *FEBS Lett.* **230**:85–89 (1988).
21. Mumby, S., I.-H. Pang, A. G. Gilman, and P. C. Sternweis. Chromatographic resolution and immunologic identification of the α_{40} and α_{41} subunits of guanine nucleotide-binding regulatory proteins from bovine brain. *J. Biol. Chem.* **263**:2020–2026 (1988).
22. Kitano, T., M. Go, U. Kikkawa, and Y. Nishizuka. Assay and purification of protein kinase C. *Methods Enzymol.* **124**:349–352 (1986).
23. Haga, K., and T. Haga. Purification of the muscarinic acetylcholine receptor. *J. Biol. Chem.* **260**:7927–7935 (1985).
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. Laemmli, U. K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond.)* **227**:680–685 (1970).
26. Milligan, G., P. Gierschik, A. M. Spiegel, and W. A. Klee. The GTP-binding regulatory proteins of neuroblastoma × glioma, NG108-15, and glioma, C6, cells. *FEBS Lett.* **195**:225–230 (1986).
27. Gierschik, P., D. Sidiropoulos, A. Spiegel, and K. H. Jakobs. Purification and immunochemical characterization of the major pertussis-toxin-sensitive

guanine-nucleotide-binding protein of bovine-neutrophil membranes. *Eur. J. Biochem.* **165**:185–194 (1987).
28. Toutant, M., D. Aunis, J. Bockaert, V. Homberger, and B. Rouot. Presence of three pertussis toxin substrates and G_{α_o} immunoreactivity in both plasma and granule membranes of chromaffin cells. *FEBS Lett.* **215**:339–344 (1987).
29. Katada, T., A. G. Gilman, Y. Watanabe, S. Bauer, and K. H. Jakobs. Protein kinase C phosphorylates the inhibitory guanine-nucleotide-binding regulatory component and apparently suppresses its function in hormonal inhibition of adenylate cyclase. *Eur. J. Biochem.* **151**:431–437 (1985).
30. Fukuda, K., T. Kubo, I. Akiba, A. Maeda, M. Mishina, and S. Numa. Molecular distinction between muscarinic acetylcholine receptor subtype. *Nature (Lond.)* **327**:623–625 (1987).
31. Harden, T. K., L. I. Tanner, M. W. Martin, N. Nakahata, A. R. Hughes, J. R. Hepler, T. Evans, S. B. Masters, and J. H. Brown. Characteristics of two biochemical responses to stimulation of muscarinic cholinergic receptors. *Trends Pharmacol. Sci.* (suppl.) 14–18 (1986).
32. Schaffner, W., and C. Weissman. A rapid, sensitive, and specific method for the determination of protein in dilute solution. *Anal. Biochem.* **56**:502–514 (1973).

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