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# Characterization of the G Protein Involved in the Muscarinic Stimulation of Adenylyl Cyclase of Rat Olfactory Bulb

MARIA C. OLIANAS and PIERLUIGI ONALI

*Section on Biochemical Pharmacology, Department of Neurosciences, University of Cagliari, 09124 Cagliari, Italy*

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## SUMMARY

We investigated the identity of the G protein mediating the muscarinic stimulation of adenylyl cyclase in rat olfactory bulb membranes by examining the sensitivity of this response to selective anti-G protein antisera. Preincubation of tissue membranes with the antisera AS/7 (anti-$G_{i1/2\alpha}$), EC/2 (anti-$G_{i3\alpha}/G_{o\alpha}$), and GO/1(anti-$G_{o\alpha}$) but not with the antiserum QL (anti-$G_{q/11\alpha}$) significantly attenuated the carbachol-stimulated adenylyl cyclase activity. These antisera had no effect on the enzyme activity stimulated by the β-adrenergic agonist L-isoproterenol. On the other hand, the anti-$G_{s\alpha}$ antiserum RM/1 markedly depressed both carbachol- and L-isoproterenol-stimulated adenylyl cyclase activities. This antiserum also reduced the basal enzyme activity to a similar extent. However, different than the anti-$G_i/G_o$ antisera, the RM/1 antiserum failed to affect the carbachol-stimulated [$^{35}$S]guanosine 5′-*O*-(3-thiotriphosphate) binding to membrane G proteins, whereas it curtailed the [$^{35}$S]guanosine 5′-*O*-(3-thiotriphosphate) binding stimulated by pituitary adenylate cyclase-activating peptide. Exposure to either pertussis toxin or the anti-$G_{o\alpha}$ antiserum 9072 but not to cholera toxin or the anti-$G_{s\alpha}$ antiserum 1191 reduced the high-affinity binding of oxotremorine M to muscarinic receptors. Moreover, the labeling of a 45-kDa protein catalyzed by cholera toxin was markedly stimulated by pituitary adenylate cyclase-activating peptide but not by carbachol. These data indicate that in rat olfactory bulb membranes, muscarinic receptors interact with both $G_i$ and $G_o$ and that these G proteins mediate the stimulation of adenylyl cyclase. Although this response appears to require $G_s$ activity, no evidence was found for the direct coupling of muscarinic receptors to $G_s$.

In membranes of rat olfactory bulb, cholinergic agonists increase basal adenylyl cyclase activity and potentiate the stimulation of the enzyme elicited by other neurotransmitters, such as corticotropin-releasing hormone and vasoactive intestinal peptide (1, 2). These effects appear to be predominantly mediated by activation of a receptor pharmacologically equivalent to the m4 gene product (3, 4) and to occur independent of phospholipid hydrolysis (5). As activation of m4 muscarinic receptors generally results in inhibition of cAMP formation (6, 7), the observation of adenylyl cyclase stimulation suggests the occurrence of a new mode of receptor/effector coupling. An important step toward the understanding of the molecular mechanisms mediating this stimulatory response is the identification of the G protein that is involved. Reconstitution experiments have demonstrated that muscarinic receptors have the ability to activate different G proteins, such as $G_i$, $G_o$, $G_q$, $G_{11}$, and $G_z$ (8–11). Previously, it was shown that injection of pertussis toxin into the olfactory bulbs prevents the muscarinic stimulation of adenylyl cyclase, suggesting that G proteins of the $G_i/G_o$ family may play a role (1). However, because hormonal stimulation of adenylyl cyclase generally occurs through the activation of the stimulatory G protein $G_s$ (12), the possibility of a direct interaction of muscarinic receptor with this G protein must be considered. Recent studies have shown that in membranes of cells transfected with either the m1 or m4 muscarinic receptor gene, carbachol increases adenylyl cyclase activity, probably by promoting the coupling of the receptors to $G_s$ (13, 14).

A method widely used to investigate the receptor/G protein coupling in cell membranes uses selective antibodies raised against the carboxyl terminus of the α subunit of the G proteins ($G_\alpha$) (15). This domain has been demonstrated to be a major site of receptor/G protein interaction (16), and $G_\alpha$ carboxyl-terminal antibodies have been shown to selectively disrupt G protein activation by receptors, thus constituting a sensitive tool for identifying the G proteins mediating a specific receptor-induced signaling event (17–20). In the present study, the nature of the G proteins involved in the muscarinic stimulation of adenylyl cyclase in rat olfactory bulb was investigated by examining the ability of specific $G_\alpha$ carboxyl-terminal antisera to inhibit this response.

**ABBREVIATIONS:** PACAP, pituitary adenylate cyclase-activating peptide; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; [$^{35}$S]GTPγS, [$^{35}$S]guanosine 5′-*O*-(3-thio)triphosphate; [$^3$H]NMS, 1-[*N*-methyl-$^3$H]scopolamine methyl chloride; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; KIU, kallikrein-inactivating units; BSA, bovine serum albumin; oxo M, oxotremorine M.

## Materials and Methods

[$\alpha$-$^{32}$P]ATP (30–40 Ci/mmol), [2,8-$^{3}$H]cAMP (25 Ci/mmol), [*adenylate*-$^{32}$P]NAD (800 Ci/mmol), [$^{35}$S]GTP$\gamma$S (1426 Ci/mmol), and antisera RM/1, AS/7, EC/2, GO/1, and QL were obtained from DuPont-NEN. The antisera 1191 and 9072 were kindly provided by Dr. David Manning, University of Pennsylvania. The antiserum 1191 was specific for the carboxyl terminus of $G_{s\alpha}$, whereas the antiserum 9072 recognized sequences held in common between $G_{o1\alpha}$ and $G_{o2\alpha}$. Carboxyl-terminal peptides of $G_{s\alpha}$ (amino acids 384–394) and of $G_{i1\alpha}$ and $G_{i2\alpha}$ (amino acids 347–354) were synthesized by Gramsch Laboratories (Schwabhausen, Germany). [$^{3}$H]NMS (83 Ci/mmol) was obtained from Amersham (Buckinghamshire, UK). Recombinant $G_{i2\alpha}$ (immunoblot standard), pertussis toxin, and unlabeled GTP$\gamma$S were purchased from Calbiochem. PACAP 38 was obtained from Peninsula (Merseyside, UK). We purchased oxo M from Research Biochemical. Materials used for SDS gel electrophoresis and immunoblotting were obtained from Bio-Rad. Cholera toxin, carbachol, L-isoproterenol, and the other reagents were obtained from Sigma Chemical Co.

**Membrane preparation.** Male Sprague-Dawley rats (200–300 g) were killed by decapitation, and olfactory bulbs were homogenized by hand with a Teflon/glass tissue grinder in 10 volumes (w/v) of ice-cold buffer containing 10% (w/v) sucrose, 10 mM HEPES-NaOH, and 1 mM EGTA, pH 7.4. The homogenate was diluted 2-fold and centrifuged at 1000 × g for 5 min at 4°. The supernatant was aspirated and stored at ice-bath temperature. The pellet was resuspended, rehomogenized, and centrifuged as above. The supernatant was combined with the previous supernatant and centrifuged at 11,000 × g for 20 min at 4°. The pellet was resuspended in 40 volumes of hypotonic buffer containing all the constituents of the homogenization buffer except sucrose. The tissue was then centrifuged at 27,000 × g for 20 min at 4°, and the final pellet was resuspended in the same buffer and used immediately for the incubation with the antisera.

**Membrane treatment with antisera.** Olfactory bulb membranes (8–12 $\mu$g of protein) were incubated for 2.5 hr at ice-bath temperature with the $G_{\alpha}$ carboxyl-terminal antisera in a incubation medium containing 10 mM HEPES-NaOH, pH 7.4, 1 mM $MgCl_2$, 1 mM EGTA, 100 KIU/ml aprotinin, 100 $\mu$g/ml bacitracin, and 2 mg/ml BSA. Antiserum dilutions were prepared in incubation medium containing 5% normal rabbit serum. Control samples were incubated in the presence of this vehicle. Unless otherwise indicated, 10 $\mu$l of antiserum dilution or vehicle was mixed with 30 $\mu$l of membrane suspension. Receptor agonists were added at the end of the incubation. The final incubation volume was 50 $\mu$l.

**ADP-ribosylation of membrane G proteins by bacterial toxins.** Cholera toxin was activated by incubation at 37° for 10 min in a buffer containing 25 mM HEPES-NaOH, pH 7.4, 150 mM NaCl, 20 mM dithiothreitol, and 250 $\mu$g/ml toxin. Unless otherwise specified, the ADP-ribosylation reaction mixture (final volume 100 $\mu$l) contained 25 mM HEPES-NaOH, pH 7.4, 1 mM ATP, 100 $\mu$M GTP, 10 mM thymidine, 15 $\mu$M [$^{32}$P]NAD (6000 cpm/pmol), 10 $\mu$g of activated toxin, and 100 $\mu$g of membrane protein. Incubations were carried out at 25° for 2–3 hr. ADP-ribosylation by pertussis toxin was performed as previously described (1). The reactions were stopped by the addition of 10 volumes of ice-cold HEPES-NaOH buffer, and the samples were centrifuged at 15,000 × g for 5 min. The pellets were resuspended in a solution containing 10 mM Tris·HCl, pH 8.0, 1 mM EDTA, 1% SDS, 5% $\beta$-mercaptoethanol, and 50% glycerol (sample buffer); heated at 100° for 5 min; and subjected to SDS-PAGE using a slab gel made of 10% (w/v) acrylamide/0.25% bisacrylamide (resolving gel). The electrophoresis was performed as described by Laemmli (21). At the end of the electrophoresis, the gel were stained with Coomassie Brilliant Blue, dried, and exposed to Kodak X-Omat AR film with intensifying screen for 48–72 hr at −70°. The autoradiograms were analyzed by using a computer-assisted image-analysis system (Image Pro Plus Media Cybernetics, Silver Spring, MD).

When [$^{3}$H]NMS binding assays were to be performed, the buffers used for the membrane preparation contained 1 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride, 10 $\mu$g/ml soybean trypsin inhibitor, 100 KIU/ml aprotinin, and 30 $\mu$M unlabeled NAD substituted for [$^{32}$P]NAD in the ADP-ribosylation mixture. After incubation with either vehicle or the activated toxins, membranes were washed three times by centrifugation and resuspension in 50 mM sodium phosphate buffer.

**Adenylyl cyclase assay.** The enzyme activity was assayed by mixing the membrane suspension (8–12 $\mu$g of protein) with an equal volume of a reaction mixture containing (final concentrations) 50 mM HEPES-NaOH, pH 7.4, 2.3 mM $MgCl_2$, 0.2 mM [$\alpha$-$^{32}$P]ATP (60 cpm/pmol), 1 mM [$^{3}$H]cAMP, 0.5 mM EGTA, 1 mM 3-isobutyl-1-methylxanthine, 100 $\mu$M GTP, 5 mM phosphocreatine, 50 units/ml creatine phosphokinase, 10 $\mu$g of bacitracin, and 10 KIU of aprotinin. Immediately after mixing, the samples were incubated at 30° for 10 min. [$^{32}$P]cAMP was isolated according to the method of Salomon *et al.* (22).

**[$^{35}$S]GTP$\gamma$S binding assay.** The binding of [$^{35}$S]GTP$\gamma$S was assayed in control and antisera-treated membranes (3–5 $\mu$g of protein) by adding an equal volume of reaction mixture containing (final concentrations) 50 mM HEPES-NaOH, pH 7.4, 4 mM $MgCl_2$, 2.0 nM [$^{35}$S]GTP$\gamma$S, 1 mM EGTA, 100 mM NaCl, 50 $\mu$M GDP, and 10 KIU of aprotinin. Samples were incubated at 30° for 10 min, and the incubations were terminated by the addition of 5 ml of ice-cold buffer containing 10 mM HEPES-NaOH, pH 7.4, and 1 mM $MgCl_2$, followed by the rapid filtration through glass-fiber filters (Whatman GF/C) presoaked in the same buffer. The filters were washed two times with 5 ml of buffer, and the radioactivity trapped was determined by liquid scintillation spectrometry. Nonspecific binding was determined in the presence of 100 $\mu$M GTP$\gamma$S.

**[$^{3}$H]NMS binding.** The binding of [$^{3}$H]NMS to muscarinic receptors was assayed in 50 mM sodium phosphate buffer, pH 7.4, using 20–30 $\mu$g of membrane protein and 0.5–1.0 nM [$^{3}$H]NMS in a final volume of 1 ml. The incubation was carried out at 30° for 50 min. Preliminary experiments indicated that under these conditions the [$^{3}$H]NMS binding reached a steady state in 15–20 min, and this plateau remained stable up to 90 min. When the effects of G protein antisera were examined, the assay volume was 0.1 ml, the tissue membrane protein was 10–12 $\mu$g, and the incubation time was 20 min. To terminate the incubation, 4 ml of ice-cold 50 mM sodium phosphate buffer was added to each sample, followed by immediate filtration through GF/C glass-fiber filters presoaked in 0.5% polyethylenimine. The filters were washed twice with the same buffer and dried, and the bound radioactivity was counted by liquid scintillation. Nonspecific binding was determined in the presence of 5 $\mu$M atropine and corresponded to <3% of the total bound [$^{3}$H]NMS.

**Immunoblotting.** Olfactory bulb membranes (100–140 $\mu$g of protein/sample) were solubilized in sample buffer and subjected to SDS-PAGE. The proteins were electrophoretically transferred to a nitrocellulose sheet according to the method of Towbin *et al.* (23). The efficiency of the transfer was controlled by staining of the gel with Coomassie Brilliant Blue and by following the transfer of prestained protein standards purchased from either Sigma or Amersham. The nitrocellulose sheet was incubated with TBS buffer (10 mM Tris·HCl, 150 mM NaCl, pH 7.5) containing 3% BSA for 2 hr at room temperature. After washing with TBS, the sheet was incubated with the G protein antisera (each at 1:1000 dilution) overnight at room temperature. The antibody bound to nitrocellulose sheet was detected by alkaline phosphatase-conjugated goat anti-rabbit IgG.

Protein content was determined according to the method of Bradford (24), using BSA as the standard.

**Statistical analysis.** Results are reported as mean ± standard error. Competition curves were analyzed by a least-squares curve-fitting computer program (GraphPad, ISI, Philadelphia, PA).The goodness of fit of the data to a one- versus a two-site model was evaluated by the *F* test of the sum of squares of residuals from both fittings considering a level of significance of $p < 0.05$. The statistical significance of the difference between mean values was determined by Student's *t* test.

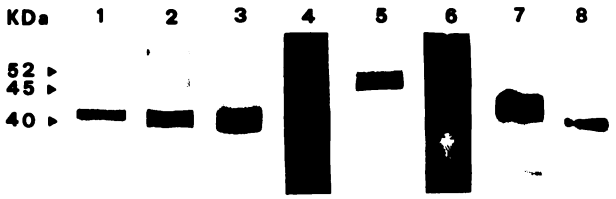


**Fig. 1.** Detection of G proteins in membranes of rat olfactory bulb. *Lanes 1–3, 5, and 7*, membrane proteins separated by SDS-PAGE and transferred to a nitrocellulose sheet were incubated with antiserum AS/7 (*lane 1*), EC/2 (*lane 2*), GO/1 (*lane 3*), RM/1 (*lane 5*), and QL (*lane 7*), each at 1:1000 dilution. *Lane 8*, immunoblotting of recombinant $G_{i2\alpha}$ standard with AS/7 antiserum. *Lanes 4 and 6*, autoradiograms of membrane proteins ADP-ribosylated with either pertussis toxin (*lane 4*) or cholera toxin (*lane 6*). The results are representative of four experiments.

## Results

**Immunodetection of G protein α subunits in olfactory bulb membranes.** Immunoblotting experiments were performed to assess whether the anti-G protein antisera recognized the presence of the corresponding protein antigen in rat olfactory bulb membranes. As shown in Fig. 1, the AS/7 antiserum, directed against the carboxyl termini of both $G_{i1\alpha}$ and $G_{i2\alpha}$ (25), detected a single immunoreactive band of 41 kDa (*lane 1*), which corresponds to the molecular mass of $G_{i1\alpha}$ (25). In the same gel run, recombinant $G_{i2\alpha}$, used as a standard, displayed a molecular mass of 40 kDa (*lane 8*). The EC/2 antiserum, which recognizes both $G_{i3\alpha}$ and $G_{o\alpha}$ (17), and the GO/1 antiserum, which is more selective for $G_{o\alpha}$ than for $G_{i3\alpha}$ (17), detected the presence of a broad protein band of 39–41 kDa (*lanes 2 and 3*, respectively), which may include both $G_{o\alpha}$ and $G_{i3\alpha}$. Moreover, the immunoreactive bands recognized by the antisera AS/7, EC/2, and GO/1 displayed an electrophoretic mobility similar to a protein band ADP-ribosylated by pertussis toxin (*lane 4*), further supporting their identification as the α subunits of $G_i/G_o$. On the other hand, the QL antiserum, which is directed against the carboxyl termini of $G_{q\alpha}$ and $G_{11\alpha}$ (26), detected two protein bands of 41.5 and 42.5 kDa (*lane 7*). The molecular masses of these proteins agree with the reported size of $G_{q\alpha}$ and $G_{11\alpha}$ (10). Finally, the antiserum RM/1, raised against a carboxyl-terminal peptide common to both $G_{s\alpha}$ and $G_{olf\alpha}$ (18), detected a major immunoreactive band of 45 kDa and a minor band of ~52 kDa (*lane 5*), which may correspond to the α subunits of the small and large forms of $G_s$, respectively, and to the α subunit of $G_{olf}$ (27). Accordingly, a protein band of 45 kDa was also the major substrate ADP-ribosylated by cholera toxin (*lane 6*), which is known to act on the α subunit of both $G_s$ and $G_{olf}$ (27).

**Effect of $G_\alpha$ carboxyl-terminal antisera on the muscarinic stimulation of adenylyl cyclase activity.** Pretreatment of rat olfactory bulb membranes with AS/7, EC/2, or GO/1 antiserum caused a concentration-dependent decrease of the adenylyl cyclase activity stimulated by a maximally effective concentration (1 mM) of carbachol in the presence of 100 μM GTP (Fig. 2). Under the same experimental conditions, the enzyme activity stimulated by a maximally effective concentration (10 μM) of the β-adrenergic agonist L-isoproterenol was not affected, whereas the basal activity assayed in the presence of 100 μM GTP was significantly reduced only by the highest concentration of the antisera. Both the EC/2 and GO/1 antisera (1:40 dilution) also failed to significantly affect the enzyme stimulation produced by a submaximal concentration (0.5 μM) L-isoproterenol (results not shown).

On the other hand, pretreatment of the membranes with the RM/1 antiserum caused a marked reduction in basal adenylyl cyclase activity and in the enzyme activities stimulated by either carbachol or L-isoproterenol (Fig. 3). Conversely, the exposure to the QL antiserum failed to affect adenylyl cyclase assayed under each condition. The inhibitory effects of the RM/1 and AS/7 antisera (each at 1:80 dilution) were reduced by 84% and 60%, respectively, after coincubation with the cognate carboxyl-terminal peptide (each at 10 μM) (results not shown).

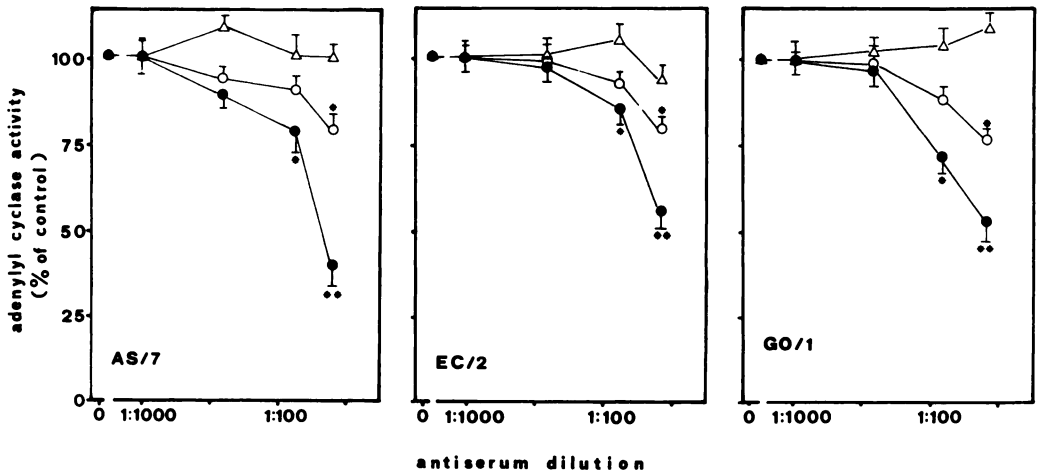


**Fig. 2.** Effect of antisera AS/7, EC/2, and GO/1 on basal (○) and carbachol-(●) and L-isoproterenol-(△) stimulated adenylyl cyclase activities. Membranes were preincubated with either normal rabbit serum (control) or the indicated antiserum dilutions. At the end of the preincubation, either vehicle, carbachol, or L-isoproterenol was added, and the adenylyl cyclase assay was immediately started. The final concentrations of carbachol and L-isoproterenol were 1 mM and 10 μM, respectively. The basal activity is that determined in the presence of 100 μM GTP, whereas the agonist-stimulated activity represents the net increase above basal activity. One hundred percent control enzyme activities (expressed as pmol of cAMP/min/mg of protein ± standard error) were basal, 110.4 ± 5.3; L-isoproterenol-stimulated, 28.2 ± 2.1; and carbachol-stimulated, 51.2 ± 4.8. Values are the mean ± standard error of four experiments for each antiserum. *, $p < 0.05$, **, $p < 0.01$ versus control by comparing the actual changes in enzyme activities.

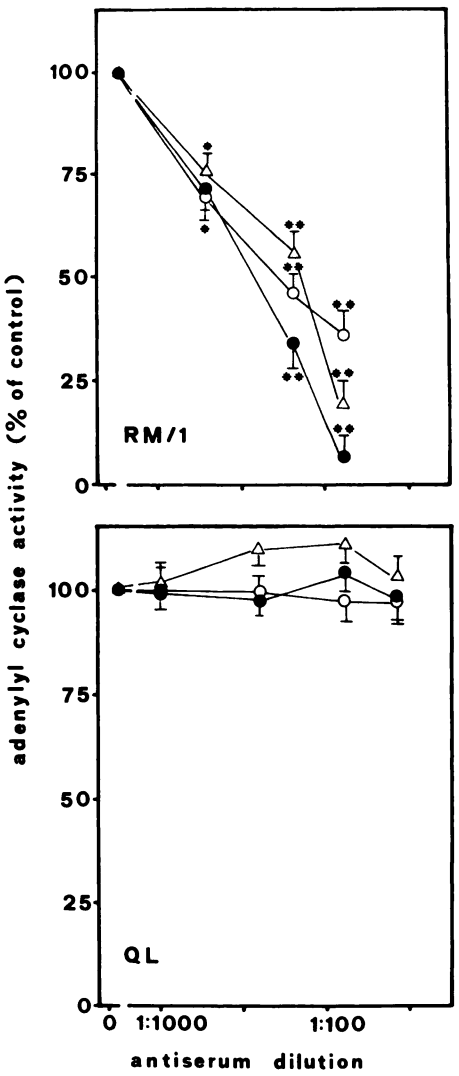


**Fig. 3.** Effect of RM/1 and QL antisera on basal (○) and carbachol-(●) and L-isoproterenol-(△) stimulated adenylyl cyclase activities. Membranes were preincubated with either normal rabbit serum (control) or the indicated antiserum dilutions. Then, either vehicle, carbachol, or L-isoproterenol was added, and the adenylyl cyclase assay was immediately started. The final concentrations of carbachol and L-isoproterenol were 1 mM and 10 μM, respectively. The agonist-stimulated activity was calculated as the net increase of enzyme activity elicited by the agonist above basal activity. One hundred percent control enzyme activities (expressed as pmol of cAMP/min/mg of protein ± standard error) were basal, 115.1 ± 7.4; L-isoproterenol-stimulated, 30.4 ± 4.7; and carbachol-stimulated, 53.1 ± 3.8. Values are the mean ± standard error of three experiments for each antiserum. *, $p < 0.05$, **, $p < 0.01$ versus control by comparing the actual changes in enzyme activities.

**Effect of $G_\alpha$ carboxyl-terminal antisera on the receptor-stimulated [$^{35}$S]GTPγS binding.** In rat olfactory bulb, carbachol (1 mM) maximally stimulated the binding of the stable GTP analogue [$^{35}$S]GTPγS to membrane G proteins by 2.5–3.0-fold. The $EC_{50}$ of this effect (3.5 ± 0.3 μM from three experiments) was close to that reported for the stimulation of adenylyl cyclase activity (0.92 μM; Ref. 28). Atropine (2 μM) completely antagonized the stimulation of [$^{35}$S]GTPγS binding by 1 mM carbachol (result not shown). As reported in Fig. 4, preincubation of rat olfactory bulb membranes with the AS/7, EC/2, and GO/1 antisera significantly reduced the carbachol-stimulated [$^{35}$S]GTPγS binding, whereas the RM/1 and QL antisera were without effect. In contrast, the RM/1 antiserum greatly reduced the stimulation of [$^{35}$S]GTPγS binding by 10 nM PACAP 38. In a separate set of experiments, PACAP 38 displayed a similar potency in stimulating [$^{35}$S]GTPγS binding and adenylyl cyclase activity ($EC_{50}$ values were 120 ± 10 and 100 ± 11 pM, respectively, from three experiments). The PACAP 38-stimulated [$^{35}$S]GTPγS binding was unaffected by membrane pretreatment with the AS/7, EC/2, GO/1, and QL antisera. The treatment with each antiserum had no significant effect on basal [$^{35}$S]GTPγS binding.

**Effect of bacterial toxins and $G_\alpha$ carboxyl-terminal antisera on oxo M displacement of [$^3$H]NMS binding.** Increasing concentrations of the muscarinic agonist oxo M yielded a biphasic displacement curve of [$^3$H]NMS bound to rat olfactory bulb membranes (Fig. 5). Computer-assisted analysis indicated that the oxo M curve was better fitted ($p < 0.05$) to a two-site competition model, with 54% and 46% of the sites displaying low and high affinity for the agonist, respectively ($EC_{50}$ values were 14.2 ± 1.7 μM and 9.5 ± 1.3 nM, respectively, from three experiments). The addition of 100 μM GTPγS to the binding assay medium markedly enhanced the $EC_{50}$ of oxo M at the high affinity site (90.5 ± 11.1 nM) without affecting that at the low affinity site (15.5 ± 1.1 μM). Pretreatment of membranes with cholera toxin did not significantly change the affinities of oxo M for the two binding sites. $EC_{50}$ values were 16.1 ± 1.2 μM and 11.± 1.0 nM for vehicle-treated membranes and 20.0 ± 1.8 μM and 12.1 ± 1.5 nM for cholera toxin-treated membranes, respectively. In these membranes, the effectiveness of cholera toxin-induced ADP-ribosylation of $G_s$ was documented by a 2.5–3-fold increase of adenylyl cyclase activity (result not shown). Conversely, exposure to pertussis toxin significantly enhanced the $EC_{50}$ of oxo M at the high affinity site (vehicle-treated, 5.8 ± 0.7 nM; toxin-treated, 25.1 ± 2.8 nM; results from three experiments; $p < 0.05$), with no change in that at the low affinity site (vehicle-treated, 12.1 ± 0.8 μM; toxin-treated, 13.2 ± 1.2 μM).

As shown in Fig. 6, the displacement of [$^3$H]NMS binding by 30 nM oxo M was significantly reduced by pretreatment of the membranes with the antiserum 9072, which recognizes the carboxyl terminus of $G_{o\alpha}$, but not by the antiserum 1191, directed against the carboxyl terminus of $G_{s\alpha}$.

**Effect of carbachol and PACAP 38 on G protein ADP-ribosylation by cholera toxin.** Incubation of rat olfactory bulb membranes with activated cholera toxin, low NAD concentrations (0.1–0.2 μM [$^{32}$P]NAD), and no exogenous GTP resulted in the predominant labeling of two bands, with one major band migrating at 45 kDa and a minor band of 42.5 kDa (Fig. 7). The addition of 1 mM carbachol did not affect the incorporation of [$^{32}$P]ADP-ribose into either band. On the other hand, PACAP 38 (10 nM) stimulated the labeling of the 45 kDa band by 2-fold. The incorporation of [$^{32}$P]ADP-ribose into the 42.5-kDa band was not significantly increased by PACAP 38.

## Discussion

In the present study, we attempted to identify the G proteins involved in the muscarinic stimulation of adenylyl cyclase in membranes of rat olfactory bulb by using a panel of anti-G protein antisera capable of interfering with receptor/G

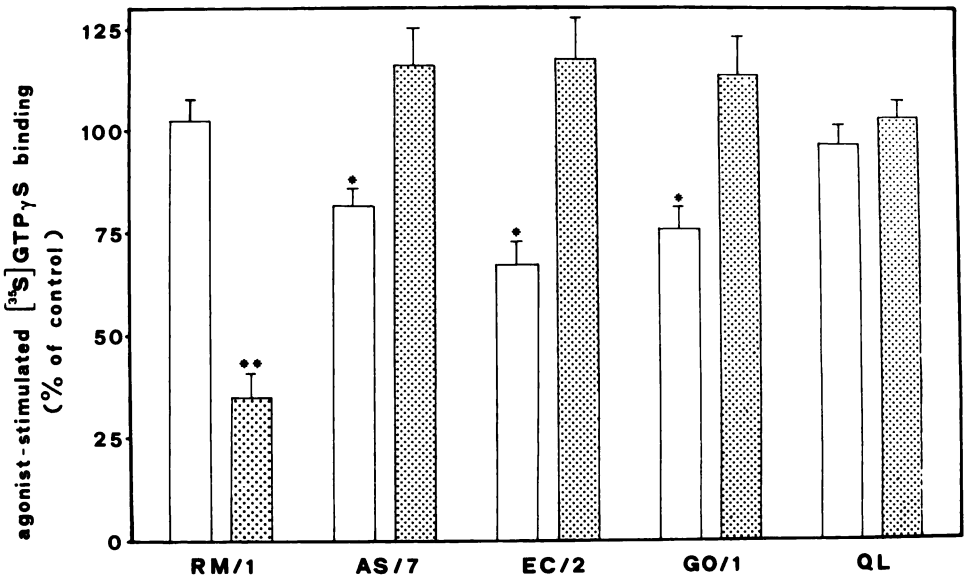


**Fig. 4.** Effects of anti-G protein antisera on [$^{35}$S]GTPγS binding stimulated by 10 μM carbachol (*open columns*) or 10 nM PACAP 38 (*shaded columns*). Membranes were preincubated with either normal rabbit serum (control) or the RM/1 (1:80), AS/7 (1:40) EC/2 (1:40), GO/1 (1:30), or QL (1:40) antiserum. Vehicle or agonist was then added, and the [$^{35}$S]GTPγS binding assay was immediately started and continued for 10 min at 30°. The concentration of [$^{35}$S]GTPγS was 2.0 nM. Agonist-stimulated [$^{35}$S]GTPγS binding was calculated as the net increase elicited by the agonist above basal binding (1.15 ± 0.05 pmol/mg of protein). One hundred percent control [$^{35}$S]GTPγS binding values (expressed as pmol/mg protein ± standard error) were carbachol-stimulated, 1.02 ± 0.11; and PACAP 38-stimulated, 0.25 ± 0.02. Values are the mean ± standard error of three experiments for each antiserum. *, $p < 0.05$, **, $p < 0.01$ versus control by comparing the actual changes in agonist-dependent [$^{35}$S]GTPγS binding.

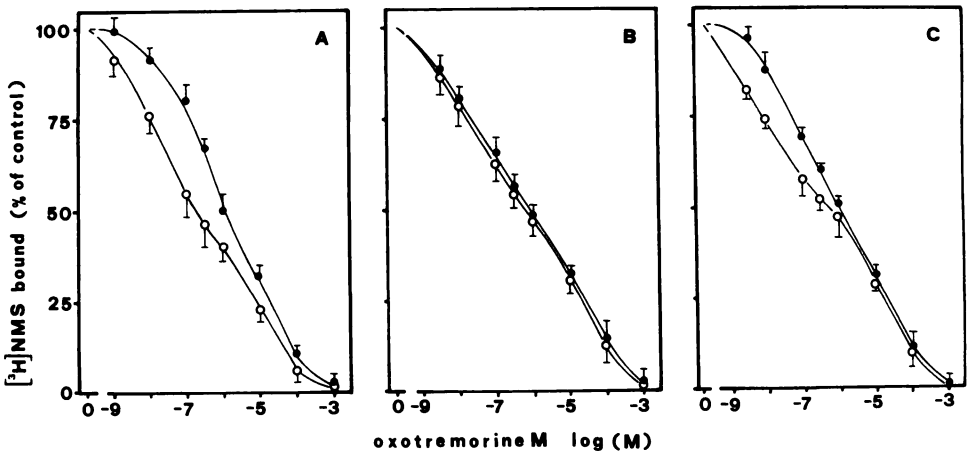


**Fig. 5.** Displacement of [$^{3}$H]NMS binding by oxo M in rat olfactory bulb membranes. Membranes were incubated with 0.5 nM [$^{3}$H]NMS in the presence of the indicated concentrations of oxo M for 50 min at 30°. A, Assays were performed in the absence (○) and presence (●) of 100 μM GTPγS. B, oxo M competition curve in vehicle-(○) and cholera toxin-(●) treated membranes. C, oxo M competition curve in vehicle-(○) and pertussis toxin-(●) treated membranes. One hundred percent control binding was A, 1.86 ± 0.11 and 1.80 ± 0.08 pmol/mg of protein with and without GTPγS, respectively; B, 1.70 ± 0.09 and 1.61 ± 0.10 pmol/mg of protein for vehicle- and cholera toxin-treated membranes, respectively; and C, 1.79 ± 0.12 and 1.70 ± 0.09 pmol/mg of protein for vehicle- and pertussis toxin-treated membranes, respectively. Values are the mean ± standard error of three experiments.

protein interactions. As demonstrated by the results of immunoblotting experiments, the antisera recognized the appropriate protein antigen in the membranes. The observation of the presence in the rat olfactory bulb of $G_i$, $G_o$, $G_s/G_{olf}$, and $G_q/G_{11}$ is consistent with the results reported by others (29–31). When the antisera were used to investigate the nature of the G protein involved in the muscarinic stimulation of adenylyl cyclase, it was found that the anti-$G_{i\alpha}/G_{o\alpha}$ antisera were able to impair the muscarinic stimulatory response in a selective and specific manner. At concentrations effective in reducing the carbachol stimulation of adenylyl cyclase, these antisera failed to affect the enzyme stimulation elicited by L-isoproterenol. The inhibition produced by the AS/7 antiserum was counteracted by coincubation with the carboxyl-terminal peptide common to $G_{i1\alpha}$ and $G_{i2\alpha}$, thus ruling out the possibility of unspecific effects. Moreover, the exposure to the QL antiserum was without effect on either basal or receptor-stimulated enzyme activities. The sensitivity to the AS/7, EC/2, and GO/1 antisera and the lack of inhibition by the QL antiserum indicated that $G_i/G_o$, but not $G_q/G_{11}$, mediated the muscarinic stimulation of adenylyl cyclase in the olfactory bulb. These results were in line with the previous observation (1) that the muscarinic stimulation of adenylyl cyclase was markedly reduced by pretreatment of olfactory

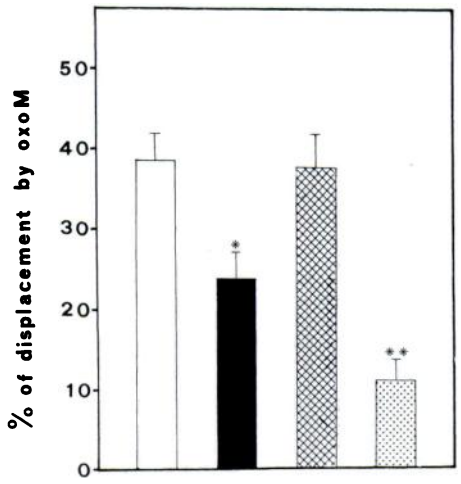


**Fig. 6.** Effect of antisera 1191 (anti-$G_{s\alpha}$) and 9072 (anti-$G_{o\alpha}$) on oxo M antagonism of [$^3$H]NMS binding. Membranes (10–12 $\mu$g of protein) were preincubated for 60 min at 30° with either normal rabbit serum (*open column*), antiserum 9072 (1:20 dilution; *filled column*), antiserum 1191 (1:20 dilution; *cross-hatched column*), or 100 $\mu$M GTP$\gamma$S (*shaded column*). [$^3$H]NMS with or without oxo M was then added, and the incubation was continued for 20 min. The final concentrations of [$^3$H]NMS and oxo M were 2.0 and 30 nM, respectively. The antagonism of oxo M is reported as percent reduction of control [$^3$H]NMS binding. Control [$^3$H]NMS binding values (expressed as pmol/mg of protein) were normal rabbit serum, 2.95 ± 0.15; normal rabbit serum plus GTP$\gamma$S; 3.09 ± 0.14; and membranes treated with antisera 1191 and 9072, 3.23 ± 0.18 and 3.09 ± 0.14, respectively. Values are the mean ± standard error of four experiments. *, $p < 0.05$, **, $p < 0.01$ versus normal rabbit serum.

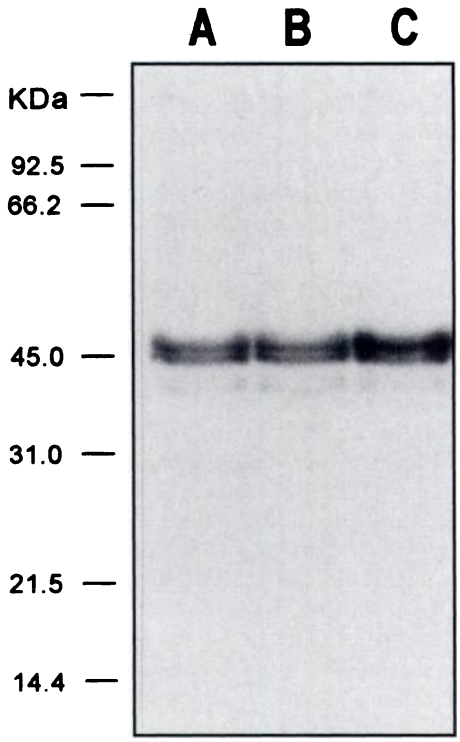


**Fig. 7.** Effect of carbachol and PACAP 38 on cholera toxin-catalyzed ADP-ribosylation of membrane G proteins. Membranes were incubated for 60 min at 25° with activated cholera toxin and [$^{32}$P]NAD in the absence (*lane A*) and presence of either 1 mM carbachol (*lane B*) or 10 nM PACAP 38 (*lane C*). At the end of the incubation, membranes were collected by centrifugation, resuspended in sample buffer, and subjected to SDS-PAGE. Gels were stained, dried, and exposed to Kodak X-Omat AR film for 48 hr. The autoradiogram is representative of three experiments yielding similar results.

bulb with pertussis toxin, which selectively ADP-ribosylates $G_i/G_o$ proteins and uncouples them from receptors (15).

However, the carbachol stimulation of adenylyl cyclase was also significantly reduced by membrane treatment with the anti-$G_s/G_{olf}$ antiserum RM/1. This effect appeared to be specific because it was counteracted by coincubation with the cognate carboxyl-terminal peptide and occurred at the same antiserum dilutions that prevented the $\beta$-adrenergic stimulation of the enzyme. These results indicated that $G_{s\alpha}$ participated in the muscarinic stimulation of adenylyl cyclase. The possibility of a direct interaction of muscarinic receptors with $G_{s\alpha}$ in rat olfactory bulb membranes was investigated by three different methods.

First, the muscarinic-stimulated [$^{35}$S]GTP$\gamma$S binding was used as a direct index of receptor/G protein interaction. The carbachol stimulation of [$^{35}$S]GTP$\gamma$S binding was significantly attenuated by membrane pretreatment with anti-$G_i/G_o$ antisera but not with the RM/1 antiserum. The adequacy of this treatment was evaluated by examining the effect of the RM/1 antiserum on the [$^{35}$S]GTP$\gamma$S binding stimulated by activation of the PACAP receptor, which is effectively coupled to stimulation of adenylyl cyclase in rat olfactory bulb (32). It was found that the RM/1 antiserum, but not the anti-$G_i/G_o$ antisera, caused a significant inhibition of the PACAP-stimulated [$^{35}$S]GTP$\gamma$S binding. Thus, these data, while clearly indicating the selective interaction of PACAP receptors with $G_s$, failed to demonstrate the occurrence of a direct coupling of muscarinic receptor to this G protein. With the exception of the QL antiserum, the anti-G protein antisera failed to affect basal [$^{35}$S]GTP$\gamma$S binding but significantly reduced the basal adenylyl cyclase activity. This differential effect was particularly evident with the RM/1 antiserum. A possible explanation of this discrepancy is that basal [$^{35}$S]GTP$\gamma$S binding represents the interaction of [$^{35}$S]GTP$\gamma$S with a large pool of nucleotide binding proteins of which only a small proportion may control adenylyl cyclase activity and can be affected by the antiserum treatment. Moreover, it should be considered that to detect an agonist stimulation, the basal [$^{35}$S]GTP$\gamma$S binding had to be reduced by the addition of GDP and NaCl (33). It is conceivable that under these assay conditions, a further inhibition caused by the anti-G protein antisera may not be detected.

Second, we examined the ability of cholera and pertussis toxins to affect the displacement of [$^3$H]NMS binding by the muscarinic agonist oxo M. For G protein-coupled receptors, reconstitution studies have demonstrated that the high affinity agonist binding state is dependent on the formation of a receptor/G protein complex and that receptor/G protein uncoupling induced either by guanine nucleotide or alterations of the $G_\alpha$ carboxyl terminus decreases agonist affinity for the receptors (8, 34). Pertussis toxin decreases the high affinity agonist binding state by uncoupling the receptors from $G_i/G_o$ as a result of ADP ribosylation of a cysteine residue in the carboxyl terminus of the $\alpha$ subunits of the G proteins (15). Conversely, cholera toxin, by inhibiting GTPase activity of $G_{s\alpha}$ (35), promotes the stable binding of GTP and may induce receptor/G protein dissociation. For example, in F-11 neuroblastoma-sensory neuron hybrid cells, cholera toxin treatment has been found to decrease high affinity opioid agonist binding, thus revealing a coupling of

opioid receptors to $G_{s\alpha}$ (36). In rat olfactory bulb membranes, oxo M displaced [$^3$H]NMS binding in a biphasic manner, indicating the presence of a high and a low affinity binding site for the agonist. The addition of GTPγS markedly reduced the potency of oxo M in competing at the high affinity site, indicating that this site represented the G protein-coupled state of the muscarinic receptors. Treatment of the membranes with cholera toxin did not significantly affect the affinity of oxo M for either the high or low affinity site. In contrast, pertussis toxin treatment modified the oxo M competition curve in a manner similar to GTPγS in that it decreased the potency of the agonist for the high affinity site. We also examined whether antisera 1191 and 9072, raised against the carboxyl terminus of $G_{s\alpha}$ and $G_{o\alpha}$, respectively, could differentially affect the high affinity binding of oxo M. We found that membrane pretreatment with the antiserum 9072, but not with the antiserum 1191, resulted in a significant reduction of the inhibition of [$^3$H]NMS binding by oxo M. Collectively, this set of data supported the idea that in membranes of rat olfactory bulb muscarinic receptors interacted with $G_i/G_o$ but not with $G_s$.

Finally, we examined the effect of muscarinic receptor stimulation on the ADP-ribosylation of membrane G proteins catalyzed by cholera toxin. Previous studies have shown that in the absence of GTP, agonist activation of $G_s$-coupled receptors enhanced the incorporation of [$^{32}$P]ADP-ribose into $G_{s\alpha}$ (37, 38). It has been proposed that in cell membranes lacking GTP, the agonist-occupied receptors induce the release of GDP from $G_{s\alpha}$ and favor the formation of a nucleotide-depleted, receptor-coupled conformation of the G protein, which becomes a better substrate for cholera toxin (37). We therefore sought to use this response as a means of investigating the possible coupling of muscarinic receptor to $G_s$. In agreement with previous results (38), in the presence of a low NAD concentration and without exogenous GTP, the major subtrates ADP-ribosylated by cholera toxin were two protein bands of 42.5 and 45 kDa. These bands likely represented two forms of $G_{s\alpha}$ with distinct electrophoretic mobility due to differing incorporation of multiple ADP-ribose molecules (38). The addition of carbachol failed to alter the labeling of these two proteins, whereas stimulation of PACAP receptors markedly enhanced the incorporation of [$^{32}$P]ADP-ribose into the 45-kDa cholera toxin substrate. Thus, this type of experiment also yielded negative results regarding to the occurrence of a $G_s$-coupled muscarinic receptor in rat olfactory bulb membranes.

Despite several lines of evidence indicating that a direct interaction of muscarinic receptors with $G_s$ was unlikely, the sensitivity of the carbachol stimulation of adenylyl cyclase to the blocking effect of the RM/1 antiserum strongly indicated that $G_s$ was somehow involved in this response. It is possible that $G_s$ activation of adenylyl cyclase was necessary for the muscarinic stimulation to occur. Unlike the other antisera, the RM/1 antiserum caused a marked decrease in the basal enzyme activity, possibly due to an impaired GTP-induced activation of $G_s$. This possibility is consistent with previous studies reporting that antisera directed against the carboxyl terminus of $G_{s\alpha}$ not only uncoupled receptors from $G_s$ but also inhibited direct activation of $G_s$ by either guanine nucleotides or fluoride (18, 39). The requirement of $G_s$ activity is in line with the observation that activation of $G_s$-coupled receptors by either corticotropin-releasing hormone or vasoactive intestinal peptide enhanced the muscarinic stimulation of adenylyl cyclase (2) and further supports the idea that this response may results from the action of multiple G proteins on type II adenylyl cyclase (40). This form of adenylyl cyclase, which is expressed in rat olfactory bulb (41), has been shown to be stimulated by βγ subunits of G proteins, a response markedly amplified by $G_{s\alpha}$ (42). It is possible that by activating $G_i/G_o$, muscarinic receptors promote the release of βγ subunits, which in turn stimulate type II adenylyl cyclase in concert with $G_{s\alpha}$. The analysis of *in situ* coupling of muscarinic receptors to G protein conducted in the present study supports this mechanism and differentiates the muscarinic stimulation of olfactory bulb adenylyl cyclase occurring in native membranes from the $G_s$-mediated increase of the enzyme activity described in cells expressing cloned muscarinic receptors in high density (13).

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**References**

1. Olianas, M. C., and P. Onali. Properties of muscarinic-stimulated adenylate cyclase activity in rat olfactory bulb. *J. Neurochem.* **58:**1723–1729 (1992).
2. Olianas, M. C., and P. Onali. Synergistic interaction of muscarinic and opioid receptors with Gs-linked neurotransmitter receptors to stimulate adenylyl cyclase activity of rat olfactory bulb. *J. Neurochem.* **61:**2183–2190 (1993).
3. Olianas, M. C., and P. Onali. Muscarinic stimulation of adenylate cyclase activity of rat olfactory bulb: II. Characterization of the antagonist sensitivity and comparison with muscarinic inhibitions of the enzyme in the striatum and heart. *J. Pharmacol. Exp. Ther.* **259:**680–686 (1991).
4. Onali, P., A. J. Aasen, and M. C. Olianas. Antagonism by (R)- and (S)-trihexyphenidyl of muscarinic stimulation of adenylyl cyclase in rat olfactory bulb and inhibition in striatum and heart. *Br. J. Pharmacol.* **113:**775–780 (1994).
5. Olianas, M. C., and P. Onali. Stimulation of phosphoinositide hydrolysis by muscarinic receptor activation in the rat olfactory bulb. *Biochem. Pharmacol.* **45:**281–287 (1993).
6. Peralta, E. G., A. Ashkenazi, J. W. Winslow, J. Ramachandran, and D. Capon. Differential regulation of PI hydrolysis and adenylyl cyclase by muscarinic receptor subtypes. *Nature (Lond.)* **334:**434–437 (1988).
7. Hulme, E. C., N. J. M. Birdsall, and N. J. Buckley. Muscarinic receptor subtypes. *Ann. Rev. Pharmacol. Toxicol.* **30:**633–673 (1990).
8. Tota, M. R., K. R. Kahler, and M. I. Schimerlik. Reconstitution of the purified atrial muscarinic acetylcholine receptor with the purified porcine atrial inhibitory guanine nucleotide binding protein. *Biochemistry* **26:**8175–8182 (1987).
9. Florio, V. A., and P. C. Sternweis. Mechanisms of muscarinic receptor action on Go in reconstituted phospholipid vesicles. *J. Biol. Chem.* **264:**3909–3915 (1989).
10. Parker, E. M., K. Kameyama, T. Higashijima, and E. M. Ross. Reconstitutively active G protein-coupled receptors purified from baculovirus-infected insect cells. *J. Biol. Chem.* **266:**519–527 (1991).
11. Berstein, G., J. L. Blank, A. V. Smrcka, T. Higashijima, P. C. Sternweis, J. H. Exton, and E. M. Ross. Reconstitution of agonist-stimulated phosphatidylinositol 4:5-biphosphate hydrolysis using purified m1 muscarinic receptor, $G_{q/11}$ and phospholipase C-β1. *J. Biol. Chem.* **267:**8081–8088 (1992).
12. Birnbaumer, L., J. Abramowitz, and A. M. Brown. Receptor-effector coupling by G proteins. *Biochim. Biophys. Acta* **1031:**163–224 (1990).
13. Dittman A. H., J. P. Weber, T. R. Hinds, E.-J. Choi, J. C. Migeon, N. M. Nathanson, and D. R. Storm. A novel mechanism for coupling of m4 muscarinic acetylcholine receptors to calmodulin-sensitive adenylyl cyclases: crossover from G protein-coupled inhibition to stimulation. *Biochemistry* **33:**943–951 (1994).
14. Gurwitz, D., R. Haring, E. Heldman, C. M. Fraser, D. Manor, and A. Fisher. Discrete activation of transduction pathways associated with acetylcholine m1 receptor by several muscarinic ligands. *Eur. J. Pharmacol.* **267:**21–31 (1994).
15. Spiegel, A. M., A. Shenker, and L. S. Weinstein. Receptor-effector coupling by G proteins: implications for normal and abnormal signal transduction. *Endocr. Rev.* **13:**536–565 (1992).

16. Conklin, R. B., and H. R. Bourne. Structural elements of Gα subunits that interact with Gβγ, receptors, and effectors. *Cell* **73**:631–641 (1993).
17. Simonds, W. F., P. K. Goldsmith, J. Codina, C. G. Unson, and A. M. Spiegel. Gi2 mediates α2-adrenergic inhibition of adenylyl cyclase in platelet membranes: *in situ* identification with Gα C-terminal antibodies. *Proc. Natl. Acad. Sci. USA* **86**:7809–7813 (1989).
18. Simonds, W. F., P. K. Goldsmith, C. J. Woodard, C. G. Unson, and A. M. Spiegel. Receptor and effector interactions of Gs: functional studies with antibodies to the αs carboxyl-terminal peptide. *FEBS Lett.* **249**:189–194 (1989).
19. McKenzie, F. R., and G. Milligan. δ-Opioid-receptor-mediated inhibition of adenylate cyclase is transduced specifically by the guanine-nucleotide-binding protein $G_i2$. *Biochem. J.* **267**:391–398 (1990).
20. Gutowski, S., A. Smrcka, L. Nowak, D. Wu, M. Simon, and P. C. Sternweis. Antibodies to the αq subfamily of guanine nucleotide-binding regulatory protein α subunits attenuate activation of phosphatidylinositol 4,5-biphosphate hydrolysis by hormones. *J. Biol. Chem.* **266**:20519–20524 (1991).
21. Laemmli, U. K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond.)* **227**:680–685 (1970).
22. Salomon, Y., C. Londos, and M. Rodbell. A highly sensitive adenylate cyclase assay. *Anal. Biochem.* **58**:541–548 (1974).
23. Towbin, H., T. Staehelin, and J. Gordon. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci. USA* **76**:4350–4354 (1979).
24. Bradford, M. M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**:248–254 (1976).
25. Goldsmith, P, K. Rossiter, A. Carter, W. Simonds, C. G. Unson, R. Vinitsky, and A. M. Spiegel. Identification of the GTP-binding protein encoded by $G_{i3}$ complementary DNA. *J. Biol. Chem.* **263**:6476–6479 (1988).
26. Shenker, A. P. Goldsmith, C. G. Unson, and A. M. Spiegel. The G protein coupled to the thromboxane $A_2$ receptor in human platelets is a member of the novel Gq family. *J. Biol. Chem.* **266**:9309–9313.
27. Jones, D. T., S. B. Masters, H. R. Bourne, and R. R. Reed. Biochemical characterization of three stimulatory GTP-binding proteins. *J. Biol. Chem.* **265**:2671–2676 (1990).
28. Olianas, M. C., and P. Onali. Muscarinic stimulation of adenylate cyclase activity of rat olfactory bulb: I. Analysis of agonist sensitivity. *J. Pharmacol. Exp. Ther.* **259**:673–679 (1991).
29. Asano, T., H. Shinohara, R. Morishita, and K. Kato. Immunochemical and immunohistochemical localization of the G protein $G_{i1}$ in rat central nervous tissues. *J. Biochem.* **108**:988–994 (1990).
30. Drinnan, S. L., B. T. Hope, T. P. Snutch, and S. R. Vincent. $G_{olf}$ in the basal ganglia. *Mol. Cell. Neurosci.* **2**:66–70 (1991).
31. Milligan, G. Regional distribution and quantitative measurement of the phosphoinositidase C-linked guanine nucleotide binding proteins $G_{11}α$ and $G_{qα}$ in rat brain. *J. Neurochem.* **61**:845–851 (1993).
32. Cauvin, A., P. Robberecht, P. De Neef, P. Gourlet, A. Vandermeers, M.-C. Vandermeers-Piret, and J. Christophe. Properties and distribution of receptors for pituitary adenylate cyclase activating peptide (PACAP) in rat brain and spinal cord. *Regul. Pept.* **35**:161–173 (1991).
33. Wieland, T., and K. H. Jakobs. Measurement of receptor-stimulated guanosine 5′-*O*-(γ-thio)trisphosphate binding by G proteins. *Methods Enzymol.* **237**:3–13 (1994).
34. Caron, M. G., R. A. Cerione, J. L. Benovic, B. Strulovici, C. Staniszewski, R. J. Lefkowitz, J. Codina-Salada, and L. Birnbaumer. Biochemical characterization of the adrenergic receptors: affinity labeling, purification and reconstitution studies. *Adv. Cyclic Nucleotide Protein Phosphorylation Res.* **19**:1–12 (1985).
35. Cassel, D., and Z. Selinger. Mechanism of adenylate cyclase activation by cholera toxin: inhibition of GTP hydrolysis at the regulatory site. *Proc. Natl. Acad. Sci. USA* **74**:3307–3311 (1977).
36. Cruciani, R. A., B. Dvorkin, S. A. Morris, S. M. Crain, and M. H. Makman. Direct coupling of opioid receptors to both stimulatory and inhibitory guanine nucleotide-binding proteins in F-11 neuroblastoma-sensory neuron hybrid cells. *Proc. Natl. Acad. Sci. USA* **90**:3019–3023 (1993).
37. Bornancin, F., Y. Audigier, and M. Chabre. ADP-ribosylation of $G_s$ by cholera toxin is potentiated by agonist activation of β-adrenergic receptors in the absence of GTP. *J. Biol. Chem.* **268**:17026–17029 (1993).
38. Ogino, Y., C. M. Fraser, and T. Costa. Selective interaction of $β_2$- and $α_2$-adrenergic receptors with stimulatory and inhibitory guanine nucleotide-binding proteins. *Mol. Pharmacol.* **42**:6–9 (1992).
39. Nair, B. G., B. Parikh, G. Milligan, and T. B. Patel. $G_sα$ mediates epidermal growth factor-elicited stimulation of rat cardiac adenylate cyclase. *J. Biol. Chem.* **265**:21317–21322 (1990).
40. Olianas, M. C., and P. Onali. Activation of opioid and muscarinic receptors stimulates basal adenylyl cyclase but inhibits $Ca^{2+}$/calmodulin- and forskolin-stimulated enzyme activities in rat olfactory bulb. *J. Neurochem.* **63**:161–168 (1994).
41. Feinstein, P. G., K. A. Schrader, H. A. Bakalyar, W.-J. Tang, J. Krupinski, A. G. Gilman, and R. R. Reed. Molecular cloning and characterization of a $Ca^{2+}$/calmodulin-insensitive adenylyl cyclase from rat brain. *Proc. Natl. Acad. Sci. USA* **88**:10173–10177 (1991).
42. Tang, W.-J., and A. G. Gilman. Type-specific regulation of adenylyl cyclase by G protein βγ subunits. *Science (Washington D. C.)* **254**:1500–1503 (1992).

**Send reprint requests to:** Maria C. Olianas, Ph.D., Section on Biochemical Pharmacology, Department of Neurosciences, via Porcell 4, 09124 Cagliari, Italy.