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21. Liposomes were prepared from a mixture of phosphatidylserine and phosphatidylcholine (dipalmitoyl, Sigma) (1:1) by extrusion under argon pressure through a membrane with a 100-nm pore size. The membrane-binding assays contained 200 to 500 μg of phospholipid, 100 mM KCl, 40 mM 3-(*N*-morpholino)propanesulfonic acid (MOPS; pH 7.5), 5 mM $MgCl_2$, 1 mM β-mercaptoethanol, 2 mM Ca^{2+}-EGTA buffer (*28*), and 10 μg of retinal recoverin.
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24. After cleavage from recoverin, the NH_2-terminal myristoyl peptide partitions into membranes. We treated [^{3}H]myristoyl recoverin (54 μg per assay) with trypsin for 15 min (*29*) in the presence of 1 mM $CaCl_2$ to cleave off the NH_2-terminal [^{3}H]myristoyl peptide. The resulting mixture was incubated with stripped ROS membranes (100 μg of rhodopsin) and centrifuged Radioactivity in the supernatant and membranes was analyzed by scintillation counting. More than 90% of the radioactivity was found to be associated with the membranes independently of the Ca^{2+} concentration.
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29 Purified recoverin (4 μg) was digested with *N*-tosyl-L-phenylalanine chloromethyl ketone (TPCK)–treated trypsin (0.04 μg) (Sigma) for 60 min at 37°C. Reactions were stopped by the addition of 0.1 mM phenylmethysulfonyl fluoride (PMSF) and analyzed by SDS-PAGE.
30. ROS membranes from fresh or frozen (Hormel) bovine retinas were isolated on a sucrose step gradient as described (*12*). To extract Ca^{2+}-dependent membrane-binding proteins, we first homogenized bleached ROS membranes containing rhodopsin (2 mg/ml) three times on ice in 30 mM tris-HCl (pH 8.0), 100 mM NaCl, 5 mM $MgCl_2$, 1 mM β-mercaptoethanol, 0.1 mM PMSF, and 0.5 mM $CaCl_2$. The membranes were then extracted three more times with 5 mM tris-HCl (pH 8.0), 5 mM $MgCl_2$, 100 μM GTP, and 0.5 mM $CaCl_2$. The membranes were washed once more with the same buffer without GTP and then with the same buffer containing 1 mM EGTA instead of $CaCl_2$. The extracts were concentrated by pressure filtration and analyzed by SDS-PAGE.
31. Recoverin was isolated from bovine retinas either by affinity purification with a rabbit antibody to bovine recoverin (*9, 12*) or by phenyl-Sepharose chromatography (*23*) Recoverin was produced in *E. coli*, either with or without coexpression of yeast NMT, with plasmids pET11a-mr21 and pBB131 in BL21(DE3) strains, as described (*22*)
32 ROS membranes were stripped of most of their endogenous recoverin by three extractions with 5 mM tris (pH 8.0) and rhodopsin (0 1 mg/ml)
33 Purified recoverin and membranes were mixed in a 50-μl total volume containing 30 mM tris (pH 8.0), 100 mM KCl, 5 mM $MgCl_2$, 2 mM Ca^{2+}-EGTA buffer (*28*), rhodopsin (200 to 300 μg), and recoverin (20 μg) This mixture was incubated for 5 min at 37°C and centrifuged at 200,000*g* for 5 min (TLA-100 centrifuge, Beckman) The supernatants were gently aspirated, and the sedimented material was rinsed and resuspended in 45 μl of the same buffer containing 2 mM EGTA This sample was centrifuged, and the supernatants were saved for analysis Amounts of recoverin bound to the membranes were estimated by densitometric analyses of Coomassie blue–stained gels (Fig. 2)
34 To produce [^{3}H]myristoyl recoverin, we cultured *E coli* strain BL21(DE3) containing pBB131 and pET11a-mr21 in the presence of 200 μCi (50 Ci/mmol) of [^{3}H]myristic acid (Amersham) The cells were harvested and lysed, and recoverin was purified as described (*22*).
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Selectivity in Signal Transduction Determined by γ Subunits of Heterotrimeric G Proteins

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Various heterotrimeric guanine nucleotide–binding proteins have been identified on the basis of the individual subtypes of their α subunits. The βγ complexes, composed of β and γ subunits, remain tightly associated under physiological conditions and have been assumed to constitute a common pool shared among various guanosine triphosphate (GTP)–binding (G) protein heterotrimers. Particular α and β subunit subtypes participate in the signal transduction processes between somatostatin or muscarinic receptors and the voltage-sensitive L-type calcium channel in rat pituitary GH_3 cells. Among γ subunits the γ_3 subtype was found to be required for coupling of the somatostatin receptor to voltage-sensitive calcium channels, whereas the γ_4 subtype was found to be required for coupling of the muscarinic receptor to those channels.

G proteins undergo a cycle in which they switch between active and inactive states by guanine nucleotide exchange and GTP hydrolysis (*1*). The inactive G protein $\alpha_{GDP}\beta\gamma$ is stimulated by a ligand-activated receptor to exchange guanosine diphosphate (GDP) for GTP. In the active form α_{GTP} dissociates from the βγ complex. The α_{GTP} and the βγ complex are then able to interact specifically with cellular effector molecules to evoke the cellular response. Until recently it had been thought that the function of a particular G protein was solely determined by the α subunit, and no specific functions had been assigned to the β and γ subunits. The contribution of β or γ subunits to receptor-effector coupling is difficult to investigate because these subunits are functionally inactive when separated from each other. Nevertheless, the βγ complex may directly interact with effector molecules (*2–4*). Inhibition of adenyl cyclase by a stimulatory (G_s)-type G protein depends on whether the G protein is reconstituted with βγ complexes from retina or brain tissue (*5*).

Four different β polypeptide sequences are known (*6*) and all have similar sequences. Three of five identified γ cDNAs have been cloned (*7–9*). The sequence of a fourth γ cDNA, γ_4 (*10*), as well as that of a fifth γ cDNA (*11*) has been established, and additional γ subtypes may exist (*12*). Because of the apparent sequence heterogeneity in the γ subunits, functional differences of the βγ complexes have been attributed to the γ subunits (*12, 13*).

We have examined the role of individual subtypes of γ subunits in selective receptor-effector coupling. We studied the effects of γ subtype on the inhibition of voltage-sensitive Ca^{2+} channels through activation of muscarinic (M_4) or somatostatin receptors. These modulatory effects of receptor agonists are mediated by pertussis toxin–sensitive G_o proteins (*14*), and the subtypes of α_o and β subunits have specific effects in this system (*15, 16*). We detected mRNAs for γ_2, γ_3, and γ_4 subtypes in the rat pituitary tumor cell line GH_3 (Fig. 1). The γ_1 subtype has only been found in retina.

We have previously established (*15*)

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conditions for "knocking out" expression of individual subtypes of G protein subunits by nuclear microinjection of short selective antisense oligonucleotides. In the present study we injected antisense oligonucleotides that selectively hybridize with the respective mRNA for one of the γ subtypes. The effects were measured electrophysiologically with the patch-clamp method.

Carbachol (a muscarinic receptor agonist) and somatostatin were ineffective in cells that had been microinjected with an antisense oligonucleotide (γcom) directed against all known G protein γ subunit sequences (Figs. 2 and 3). Cells injected with a γ_1-selective oligonucleotide (anti-γ1.1) responded to carbachol or somatostatin in the same manner as cells not injected; a similar result was seen in cells injected with a γ_2-selective antisense oligonucleotide (anti-γ2.1). These results demonstrate that microinjection did not alter hormone responsiveness of GH_3 cells. Furthermore, they indicate that the γ_2 subunit subtype does not take part in signal transduction between somatostatin or M_4 receptors and the voltage-sensitive Ca^{2+} channel. The ineffectiveness of the injected γ_1-selective antisense oligonucleotide is in agreement with the absence of any detectable target mRNA in GH_3 cells.

In cells that had been injected with γ_3-selective antisense oligonucleotides (anti-γ3.1 and anti-γ3.2; Fig. 3), Ca^{2+} influx was no longer inhibited by somatostatin, whereas carbachol was effective. In cells that had been injected with γ_4-selective antisense oligonucleotides (anti-γ4.1 and anti-γ4.2), carbachol did not inhibit Ca^{2+} influx, whereas somatostatin was effective (Fig. 3). In conjunction with our data on the identity of the respective subtypes of α_o and β subunits that participate in this pathway (*15*, *16*), these results establish the third line of evidence demonstrating that different heterotrimeric G proteins transduce the signals from somatostatin and muscarinic receptors to voltage-sensitive Ca^{2+} channels. In the case of the somatostatin-induced signal, the G_o protein contains a γ_3 subunit; in the carbachol-induced reaction, a γ_4 subunit is required.

These findings establish the contribution of each G protein subunit (α, β, and γ) to selective receptor-effector coupling. In both of the signal transduction processes examined, a selective γ subunit subtype is apparently complexed with a specific β subunit subtype and a selective α_o subunit, giving rise to the specificity of the G proteins. Two G_o forms, distinct in all three subunits, discriminate between two distinct receptors but functionally couple to the same effector. The muscarinic receptor is coupled to a G protein consisting of $\alpha_{o1}/\beta_3/\gamma_4$, and the somatostatin receptor is coupled to a G protein consisting of $\alpha_{o2}/\beta_1/\gamma_3$. Both G proteins act to inhibit voltage-sensitive Ca^{2+} channels.

The ineffectiveness of somatostatin and carbachol in cells injected with antisense oligonucleotides to γ_3 and γ_4, respectively, probably results in the reduced synthesis of G protein subunits (*15*). Evidence for such a reduction [as occurs with α and β subunits (*15*)] has not been demonstrated for γ_3 and γ_4 subtypes (specific antibodies for these subtypes are not yet available). We propose that after nuclear microinjection of γ-selective antisense oligonucleotides the oligonu-

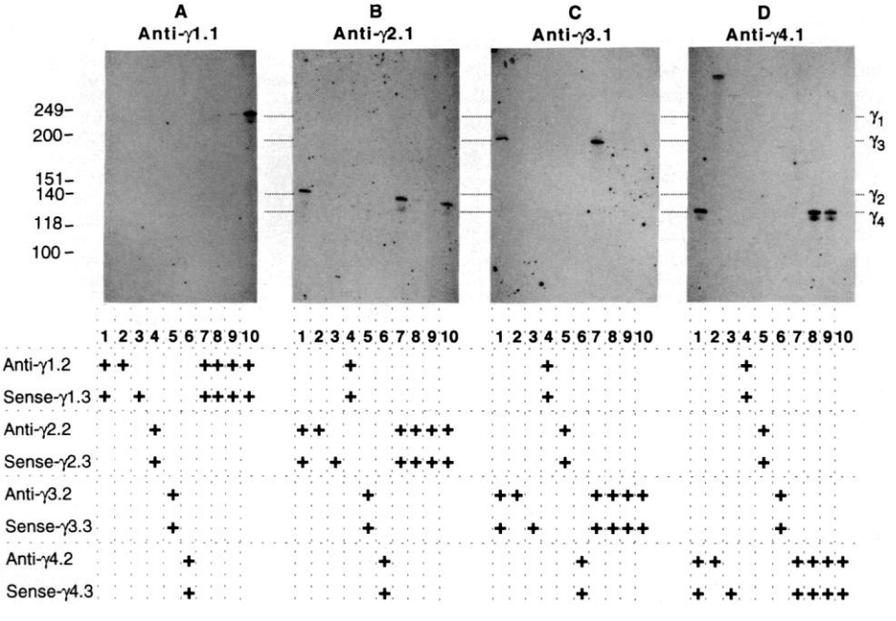


Fig. 1. Existence of mRNA encoding G protein γ subunits in GH_3 cells analyzed by polymerase chain reaction (PCR) and subsequent hybridization. Total RNA (5 μg) was randomly primed and reverse transcribed according to manufacturer's instructions (Moloney-Murine Leukemia Virus reverse transcriptase, BRL). A portion (1:100 of the reaction volume) of the cDNA was used as a template for an amplification reaction with two γ subtype–specific oligonucleotides. The PCR was performed in a reaction volume of 50 μl with 1 unit of Taq polymerase (Promega). After 35 cycles of 93°C for 60 s, 40°C for 10 s, and 72°C for 30 s in a DNA Thermal Cycler (Perkin-Elmer), the reaction products were precipitated, electrophoretically separated on a polyacrylamide gel, and transferred to a nylon membrane (Gene Screen, NEN DuPont). After cross-linking of the nucleic acids to the membrane with ultraviolet light (120 mJ, 254 nm), they were hybridized with 5' ^{32}P-labeled oligonucleotides (*22*). Autoradiograms are shown of filters that had been hybridized with oligonucleotides anti-γ1.1 (**A**), anti-γ2.1, (**B**) anti-γ3.1 (**C**), and anti-γ4.1 (**D**). The cDNAs from GH_3 cells (lanes 1 to 6), PC-12 cells (rat adrenal pheocromocytoma, lane 7), WERI-Rb-1 or Y79 cells (human retinoblastoma cell line, lanes 8 and 9, respectively), or rat retina (Clontech Laboratories, Inc.; lane 10) were amplified by PCR with primers as indicated (*23*).

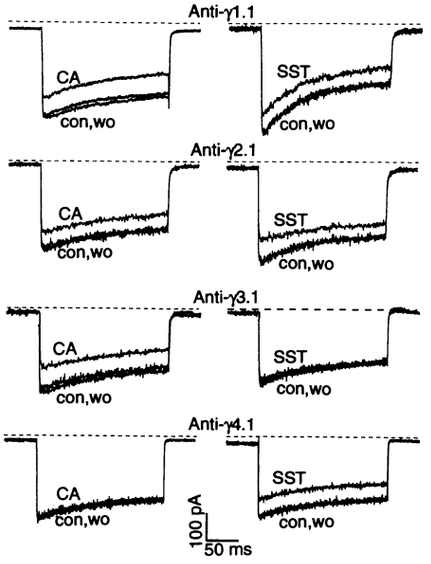
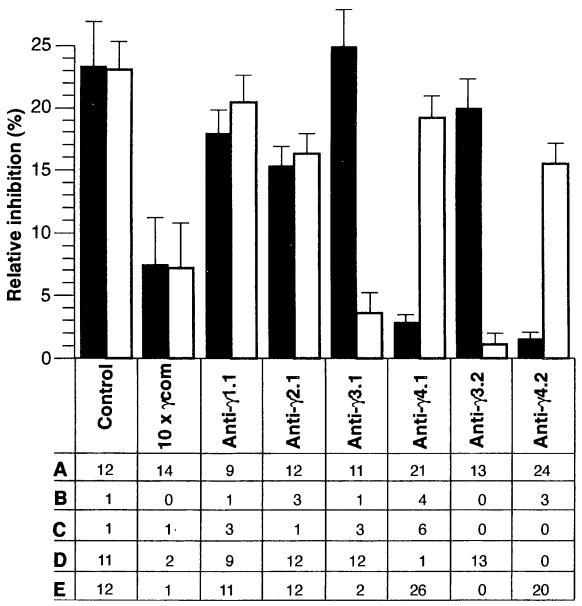


Fig. 2. Time-current recordings of the voltage-sensitive Ca^{2+} channels in GH_3 cells in the presence of carbachol (left) or somatostatin (right). Current traces for single cells are shown. Each cell was superfused with either of the hormones at about 37 hours after injection with antisense oligonucleotides anti-γ1.1, anti-γ2.1, anti-γ3.1, or anti-γ4.1 (*23*). Under voltage-clamp conditions, whole-cell Ca^{2+} currents were recorded after depolarizing pulses from −80 to 0 mV. Conditions for microinjection and patch clamping are described elsewhere (*15*). Abbreviations: con, control currents obtained before application of receptor agonist; SST, currents recorded during superfusion of cells with 1 μM somatostatin; CA, currents recorded during superfusion of cells with 10 μM carbachol; and wo, currents recorded after removal of receptor agonists.



	Control	10 x γcom	Anti-γ1.1	Anti-γ2.1	Anti-γ3.1	Anti-γ4.1	Anti-γ3.2	Anti-γ4.2
A	12	14	9	12	11	21	13	24
B	1	0	1	3	1	4	0	3
C	1	1	3	1	3	6	0	0
D	11	2	9	12	12	1	13	0
E	12	1	11	12	2	26	0	20

Fig. 3. Ca^{2+} current inhibition by receptor agonists in GH_3 cells injected with antisense oligonucleotides to mRNAs encoding γ subunit polypeptides. Whole-cell Ca^{2+} currents were measured at about 40 hours after injection of the oligonucleotide. Because of the high degeneracy of its base sequence, oligonucleotide γcom was microinjected at a concentration ten times greater than that for γ subtype–selective oligonucleotides (50 μM instead of 5 μM). Inhibition of Ca^{2+} currents by somatostatin (1 μM; open bars) or carbachol (10 μM; solid bars) is shown as a percentage of the current observed in the absence of the respective agonist (mean ± SEM). Numbers of cells tested are indicated as follows: A, cells successively treated with either of the receptor agonists (random order) with intermediate washing; B, cells treated with carbachol only; C, cells treated with somatostatin only; D, cells inhibited by carbachol by more than 10% of control currents; and E, cells inhibited by somatostatin by more than 10% of control currents. In about 10% of patched cells no Ca^{2+} currents could be activated by the applied voltage-clamp conditions.

cleotides hybridize with their target mRNAs and that the respective mRNA encoding the γ subunit is destroyed by cellular ribonuclease H or is not transferred to the cytoplasm (*17*). As a consequence, no active G protein heterotrimer can be assembled because the required γ subunit subtype is missing. This in turn abolishes the necessary receptor–G protein interaction and interrupts the cascade.

In transfection studies with the Sf9 cell–baculovirus system, β_1 and γ_3 subunits have been shown to form a functional complex that interacts with immobilized $G_{\alpha o}$ subunits (*18*). Corresponding data on the γ_4-containing complexes could not be obtained because the full-length cDNA required for such functional studies is not yet available. Functionally active complexes cannot be formed from all naturally occurring β and γ subunits. However, those containing the β_3/γ_4 and β_1/γ_3 combination found here have been neither excluded nor investigated (*18–20*). We cannot exclude more indirect mechanisms that might explain the effects of the γ-selective antisense oligonucleotides. The βγ complex may be the signaling G protein component interacting with the voltage-sensitive Ca^{2+} channel, and interruption of the signal transduction may occur at this site. Furthermore, suppression of γ_3 and γ_4 polypeptides could impair the expression of β_1 and β_3 polypeptides or α_{o2} and α_{o1} polypeptides, respectively. Moreover, expression of β and γ subunits may be linked (*21*).

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23. Sequences of the antisense oligonucleotides were as follows Anti-γ1.1 and anti-γ1.2, TCCAGCGTCACTTCTTTCTTGAGC and AATCACACAGCCTCCTTTGAG, respectively, corresponding to nucleotides 64 to 87 and 200 to 220, respectively, of the identical strand of the γ_1 gene sequence (*7*), anti-γ2 1 and anti-γ2.2, AGTTTCCTGGCTTGTGCTATGC and TTCCTTGGCATGCGCTTCAC, respectively, corresponding to nucleotides 23 to 44 and 122 to 141, respectively, of the identical strand of the γ_2 gene sequence (*8*); anti-γ3.1 and anti-γ3.2, TGCGGGCTTGCCCAATACTCAT and GTTCTCCGAAGTGGGCACAGGGGT, respectively, corresponding to nucleotides 31 to 52 and 166 to 189, respectively, of the identical strand of the γ_3 gene sequence (*9*), and anti-γ4 1 and anti-γ4 2, CTGAGGCAGCCTGGGAGACCTTC and TTTTCGGAGGCGGGCACTGGGATG, respectively, corresponding to nucleotides 37 to 59 and 108 to 129, respectively, of the identical strand of the published part of the γ_4 gene sequence (*9*) Each of the above oligonucleotides can selectively hybridize with the described γ mRNA. Sequences of the sense oligonucleotides were as follows· sense-γ1 3, AGATGCCAGTGATCAATA, corresponding to nucleotides −2 to 16 of the identical strand of the γ_1 gene sequence (*7*); sense-γ2 3, ATGGCCAGCAACAAC, corresponding to nucleotides 1 to 15 of the identical strand of the γ_2 gene sequence (*8*); sense-γ3.3, ATGAAAGGGGAGACC, corresponding to nucleotides 1 to 15 of the identical strand of the γ_3 gene sequence (*9*), and sense-γ4 3, GGAGCAGCTGAAGAT, corresponding to nucleotides 1 to 15 of the identical strand of the published part of the γ_4 gene sequence (*9*). Each of the above oligonucleotides can selectively hybridize with the complementary strand of the described γ gene sequence. The sequence of γcom was TCTKCWSMWSMYTTGGACACC, corresponding to nucleotides 97 to 117 of the identical strand of the γ_1 gene sequence (*7*). The γcom can selectively hybridize with the described γ_1 mRNA Selective primers were determined with the use of MacMolly Tetra software (Soft Gene GmbH) Abbreviations for wobbled positions are as follows W (T or A), M (A or C), Y (T or C), and S (G or C).

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