the activator only when σ^{54} is bound as holoenzyme at the promoter. The conformation of σ^{54} in the holenzyme, or the RNA polymerase subunits themselves, may also be important for DNA melting. That σ^{54} binds to promoter DNA but does not melt it is consistent with all known σ^{54}-dependent transcription systems that have a strict requirement for an activator protein[1]. Separation of promoter recognition and DNA melting functions permits $E\sigma^{54}$ to respond to many stimuli of gene expression through their influences on the activity of the activator protein. Activator dependence and assembly of the preinitiation complex in its absence strongly suggests a broad similarity to eukaryotic RNA polymerase II transcription[16,17]. □

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Different β-subunits determine G-protein interaction with transmembrane receptors

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REGULATORY GTP-binding proteins (G proteins) are membrane-attached heterotrimers (α, β, γ) that mediate cellular responses to a wide variety of extracellular stimuli[1,2]. They undergo a cycle of guanine-nucleotide exchange and GTP hydrolysis, during which they dissociate into α-subunit and $\beta\gamma$ complex[1]. The roles of G-protein α-subunits in these processes and for the specificity of signal transduction are largely established; the β- and γ-subunits are essential for receptor-induced G-protein activation and seem to be less diverse and less specific. Although the complementary DNAs for several β-subunits have been cloned[2,5–8], isolated subunits have only been studied as $\beta\gamma$ complexes[3,9–12]. Functional differences have been ascribed to the γ-subunit on the basis of extensive sequence similarity among β-subunits and apparent heterogeneity in γ-subunit sequences[13,14]. $\beta\gamma$ complexes can interact directly or indirectly with different effectors[10,11,15–20]. They seem to be interchangeable in their interaction with pertussis toxin-sensitive α-subunits[3], so we tested this by microinjecting antisense oligonucleotides into nuclei of a rat pituitary cell line to suppress the synthesis of individual β-subunits selectively. Here we show that two out of four subtypes of β-subunits tested (β_1 and β_3) are selectively involved in the signal transduction cascades from muscarinic M_4 (ref. 4) and somatostatin receptors, respectively, to voltage-dependent Ca^{2+} channels.

We have established nuclear microinjection of short selective antisense oligonucleotides as a general method to study G protein function in intact cells[21]. The effects of 'knocking out' the expression of individual G-protein α-subunits are measured in a single cell either electrophysiologically or by immunofluorescent labelling of the targeted proteins. Here we apply this approach to elucidate the role of β-subunit subtypes in the differential coupling of G proteins to the same receptor/G protein/effector systems in GH_3 cells, a rat pituitary cell line.

Cells that have been injected with the antisense oligonucleotide β-com (see Fig. 1 legend), which targets messenger RNA of all known four β-polypeptides, contain reduced amounts of immunostainable β-subunits (data not shown). The disappearance of β-subunits parallels the functional loss of inhibitory hormonal effects on Ca^{2+} currents. Ca^{2+} currents in GH_3 cells are probably due to the activation of L-type channels and are inhibited to 70–80% of control currents by carbachol and somatostatin[21]. One day after injection of the oligonucleotide β-com, neither hormone reduced the Ca^{2+} current (Fig. 1); this effect lasted for one more day. The original hormone sensitivity of the Ca^{2+} channel was restored by ~60 h after injection.

To study the question of preferential interaction between the activated receptor and G_O proteins containing a given β-subtype, GH_3 cells were injected with antisense oligonucleotides that can specifically hybridize with the mRNA of one particular β-subtype (Figs 2 and 3). Neither β_2- nor β_4-specific antisense

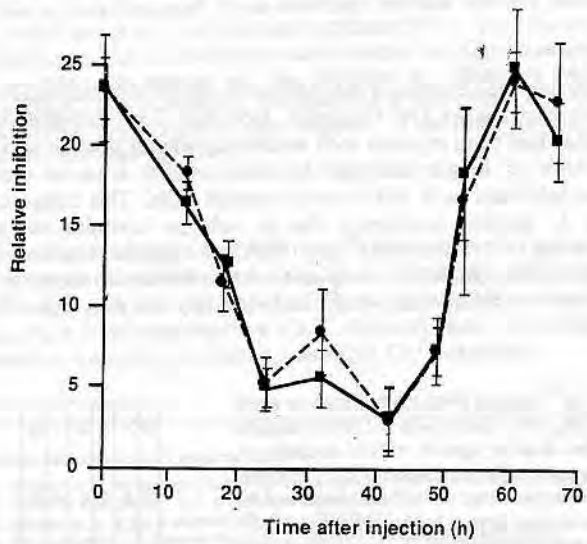

FIG. 1 Time course of Ca^{2+} current inhibition by somatostatin or carbachol in GH_3 cells. At time zero, cells were injected with antisense oligonucleotide β-com (see Methods). At the indicated time points, the Ca^{2+} current was measured in the presence of 1 μM somatostatin (dotted line) or 10 μM carbachol (solid line). Mean values with s.e.m. are shown ($n \geq 5$).
METHODS. Microinjection (about 10,000 full-length oligonucleotides per nucleus) and electrophysiological measurements were done as described[21], but during current recording cells were perfused with a solution containing 120 mM choline-Cl, 10.8 mM $BaCl_2$, 1 mM $MgCl_2$, 5.4 mM CsCl, 10 mM glucose and 10 mM tetraethylammonium-HEPES (pH 7.4 at 37 °C). Sequence of injected β-com oligonucleotide: 5'-TTGCAGTTGAAGTCGTCRTA-3', corresponding to nucleotides 825–844 of the identical strand of the β_1 gene sequence[5]. It can hybridize with the mRNAs of β_1, β_2, β_3 and β_4. Abbreviations for wobbled positions: R (G or A), M (A or C), Y (T or C), S (G or C). The oligonucleotide β-com is different from the previously used oligonucleotide anti-β[21]. Oligonucleotide β-com can hybridize perfectly with β_1, β_2, and β_3 mRNAs, and the longest continuously matching stretch in β_4 mRNA is 16 bases. The much-improved hybridization characteristics compared with oligonucleotide anti-β may explain why less β-com (<10,000 molecules, as compared with 50,000 for anti-β) is needed for effective repression of hormone responses.

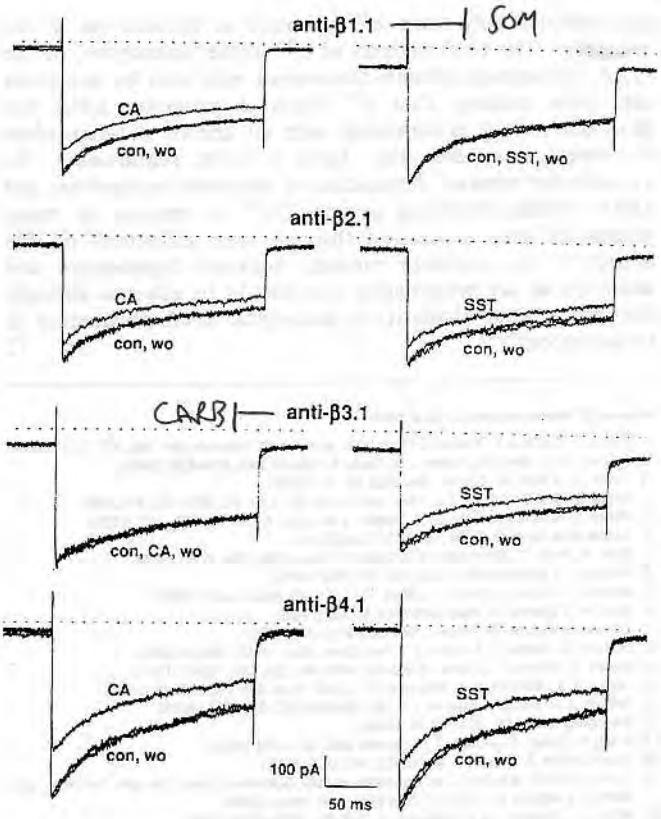

FIG. 2 Time-current recordings of the voltage-sensitive Ca^{2+} channels in GH_3 cells in the presence of carbachol (left-hand panels) or somatostatin (right-hand panels). Current traces are shown for one cell, each superfused with either hormone at 40 h after injection with antisense oligonucleotides anti-β1.1, anti-β2.1, anti-β3.1 or anti-β4.1. Under voltage-clamp conditions, whole-cell Ca^{2+} currents were recorded by depolarizing pulses from −80 to 0 mV. 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; wo, currents recorded after removal of receptor agonists. Sequences of injected oligonucleotides: anti-β1.1: 5′-GAGAGAGAGTTGC-ATCTGC-3′, corresponding to nucleotides 75–93 of the identical strand of the β_1 gene sequence[5]; anti-β2.1: 5′-GGGTCAGTGTTGAGTCCCC-3′, corresponding to nucleotides 76–94 of the identical strand of the β_2 gene sequence[6]; anti-β3.1: 5′-GGCCAGACACCAGCTCTGCC-3′, corresponding to nucleotides 90–109 of the identical strand of the β_3 gene sequence[7]; anti-β4.1: 5′-GAACCAGCGTGGCATCGTT-3′, corresponding to nucleotides 76–94 of the identical strand of the β_4 gene sequence[8]. Each can selectively hybridize with the described mRNA.

oligonucleotides prevented the inhibitory effects of somatostatin or carbachol on the Ca^{2+} channel. However, Ca^{2+} currents of cells that had been injected with antisense oligonucleotide anti-β1.1 were no longer inhibited by somatostatin, whereas carbachol inhibited as it did in non-injected cells. This suggests that a G protein containing the β_1 subtype (running on a denaturing polyacrylamide gel with an apparent relative molecular mass of 36,000 couples to the somatostatin receptor. Data are consistent with results showing that the β_{36K} subunit coprecipitates with the somatostatin receptor solubilized from rat brain[22]. In an analogous experiment, the Ca^{2+} current of cells that had been injected with antisense oligonucleotide anti-β3.1 was no longer inhibited by carbachol, whereas somatostatin was still effective. The differential receptor coupling of G_O proteins with β_1 and β_3 subunits was confirmed by injection of two other specific antisense oligonucleotides, anti-β1.2 and anti-β3.2, respectively (Fig. 3). We conclude that in these signal transduction pathways one particular $\alpha\beta\gamma$ complex out of a

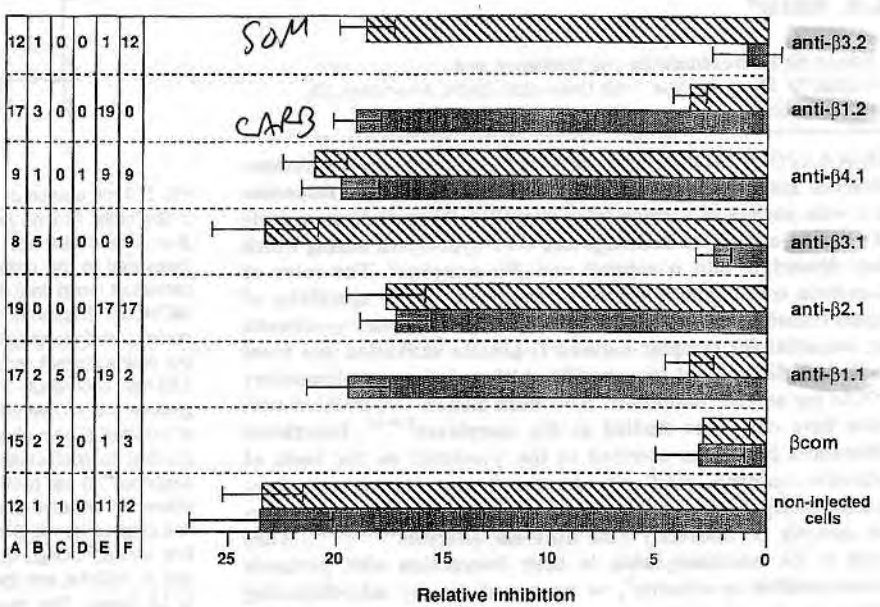

FIG. 3 Ca^{2+} current inhibition by receptor agonists in GH_3 cells injected with antisense oligonucleotides directed against mRNAs encoding β-subunit polypeptides. Whole-cell Ca^{2+} currents were measured about 40 h after injection of the oligonucleotide. Shown is the inhibition of Ca^{2+} currents by somatostatin (1 μM; hatched bars) or carbachol (10 μM; dark bars) in per cent of control current observed in the absence of the respective agonist (mean values with s.e.m. are shown). On the left-hand side are listed the numbers of cells that were used for electrophysiological measurements: A, cells successively treated with either receptor agonist (random order) with intermediate washing; B, cells treated with carbachol only; C, cells treated with somatostatin only; D, cells without measurable Ca^{2+} currents in the absence of receptor agonists; E, cells inhibited by carbachol by more than 10% of control currents; F, cells inhibited by somatostatin by more than 10% of control currents. For sequences of injected oligonucleotides βcom, anti-β1.1, anti-β2.1, anti-β3.1 and anti-β4.1, see legends to Figs 1 and 2. Sequence of anti-β1.2: 5′-TGTCCGGTAAACGTGGTCGTCT-3′, corresponding to nucleotides 526–547 of the identical strand of the β_1 gene sequence[5]. It can selectively hybridize with the described β_1 mRNA; anti-β3.2: 5′-ACGTCAGCACAGGCTTTCCT-3′, corresponding to nucleotides 64–83 of the identical strand of the β_3 gene sequence[7]. It can selectively hybridize with the described β_3 mRNA.

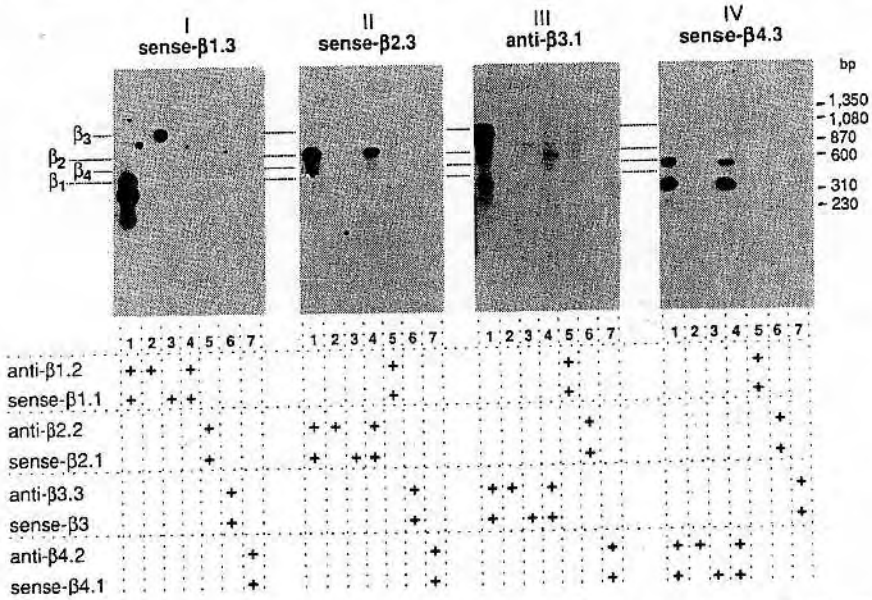

FIG. 4 Existence of mRNA encoding G protein β-subunits in GH_3 cells. mRNA (1.4 μg) isolated from GH_3 cells was reverse-transcribed with 200 U of Moloney murine leukaemia virus reverse transcriptase (BRL) in 20 μl of 0.5 mM each of dATP, dCTP, dGTP and dTTP, 1 μg random primer (dN_6), 75 mM KCl, 10 mM dithiothreitol, 3 mM $MgCl_2$, 50 mM Tris–HCl, pH 8.3, for 1 h at 37 °C. cDNA within 1/100 part reaction volume served as template for an amplification reaction using oligonucleotides that hybridize with the β_1, β_2, β_3 and β_4 gene sequences. The polymerase chain reaction (PCR) was done in a 50-μl reaction volume using 1 U of *Taq* polymerase (Promega). After 93 °C for 1.5 min, 50 °C for 10 s, 72 °C for 1 min (35 cycles) in a DNA Thermal Cycler (Perkin Elmer), reaction products were precipitated, electrophoretically separated on an agarose gel (1.5% w/v) and transferred to a nylon membrane (Gene Screen, NEN DuPont). After crosslinking the nucleic acids to the membrane with ultraviolet light (120 mJ, 254 nm), [5′-^{32}P]-labelled oligonucleotides were hybridized according to ref. 23. To achieve stringent hybridization conditions we varied the incubation temperature between 40 °C and 60 °C. Autoradiograms are shown of filters that had been hybridized with sense-β1.3 (I), sense-β2.3 (II), anti-β3.1 (III), or sense-β4.3 (IV). cDNA from GH_3 (lanes 1–3, 5–7) and RBL-2H3 cells (rat basophilic leukaemic cell line, lane 4) were amplified by PCR using primers as indicated under the panels. Sequences of oligonucleotides: for anti-β1.2 and anti-β3.1, see legends to Figs 2 and 3; sense-β1.1, sense-β1.3: 5′-GCAGATGCAACTCTCTCTC-3′ and 5′-CTCCATTTACAATCTGAAAACT-3′, corresponding to nucleotides 75–93 and 362–383 of the identical strand of the β_1 gene sequence[5], respectively; sense-β2.1, sense-β2.3: 5′-GGGGACTCAACACTGACCC-3′ and 5′-GACTCAAGGCTGCTGGTCAGCG-3′, corresponding to nucleotides 76–94 and 196–217 of the identical strand of the β_2 gene sequence[6], respectively; sense-β3: 5′-AGATTGCAGATGCCAGGAAA-3′, corresponding to nucleotides 70–89 of the identical strand of the β_3 gene sequence[7]; sense-β4.1, sense-β4.3: 5′-AACGATGCCACGCTGGTTC-3′ and 5′-CGTGGGCCGAATACAAATGC-3′, corresponding to nucleotides 76–94 and 118–137 of the identical strand of the β_4 gene sequence[8], respectively. Each of the oligonucleotides can selectively hybridize with the complementary strand of the gene described; anti-β2.2: 5′-TCGGCCCGCARGTCRAAGAGG-3′, corresponding to nucleotides 753–773 of the identical strand of the β_2 gene sequence[6]. It can selectively hybridize with the described β_2 mRNA; anti-β3.3: 5′-CCTCCTCAGTTCCAGATGTTTT-3′, corresponding to nucleotides 1,010–1,029 of the identical strand of the β_3 gene sequence[7]. It can selectively hybridize with the described β_3 mRNA; anti-β4.2: 5′-ATATCCCACAGCTTTGAGGAT-3′, corresponding to nucleotides 618–638 of the identical strand of the β_4 gene sequence[8]. It can selectively hybridize with the described β_4 mRNA.

family of G_O composite subforms interacts with the corresponding receptor.

In addition to β_1 and β_3 subunits, β_2- and β_4-subtype subunits are expressed in GH_3 cells (Fig. 4). In contrast to the former subtypes, β_2- and β_4-subunit subtypes are not involved in the carbachol- or somatostatin-induced inhibition of voltage-dependent Ca^{2+} currents.

These data show that suppression of an individual β-subunit subtype blocks a particular signal transduction pathway between an agonist-activated receptor and the assigned effector. As the G protein in its heterotrimeric form binds to the activated receptor, the β-subunit suppression caused by injected antisense oligonucleotides prevented activation of a G protein and thus transduction of the signal across the membrane. We hypothesize that the receptor recognizes and binds individual G-protein subtypes, containing different β- and γ-subunits. Despite the likely existence of other G_O protein subtypes, the somatostatin and muscarinic (M_4) receptor functionally couple via G_{02} containing the β_1 polypeptide and via G_{01} containing β_3, respectively, to the voltage-dependent Ca^{2+} channel. This hypothesis is consistent with data suggesting that the receptor recognition of G proteins is affected by specific $\beta\gamma$ complexes[11].

Although we cannot exclude a direct interaction between $\beta\gamma$ complexes and the Ca^{2+} channel, there are no indications for such effects. As β-subunit subtypes other than those targeted by the injected antisense oligonucleotide are apparently not affected, our results do not support the hypothesis of interchangeable β-subunits of $\beta\gamma$ complexes in intact cells[1], but rather suggest that the structural diversity of G proteins[2] corresponds to a functional one.

In summary, β-subunits appear to be an essential part of the specific G-protein coupling between a receptor and an effector. On the basis of these and our other[21] data, the subtypes of the G proteins involved in two signal transduction pathways in GH_3 cells can be specified as follows: muscarinic receptor → $\alpha_{01}/\beta_3/\gamma$ → voltage-sensitive Ca^{2+} channel, and somatostatin receptor → $\alpha_{02}/\beta_1/\gamma$ → voltage-sensitive Ca^{2+} channel. □

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