9. Brooks, D. A. *et al. Tectonophysics* **102**, 1-16 (1984).
10. Jordan, T. H. *J. geophys. Res.* **43**, 473-496 (1977).
11. Creager, K. C. & Jordan, T. H. *J. geophys. Res.* **89**, 3031-3049 (1984).
12. Creager, K. C. & Jordan, T. H. *J. geophys. Res.* **91**, 3573-3589 (1986).
13. Fischer, K. M., Jordan, T. H. & Creager, K. C. *J. geophys. Res.* **93**, 4773-4783 (1988).
14. Silver, P. G. & Chan, W. W. *J. geophys. Res.* **91**, 13787-13802 (1986).
15. Cormier, V. F. *J. geophys. Res.* **94**, 3006-3024 (1989).
16. Suetsugu, D. *J. Phys. Earth* **37**, 265-295 (1989).
17. Takei, Y. & Suetsugu, D. *J. Phys. Earth* **37**, 225-231 (1989).
18. Dziewonski, A. M. *J. geophys. Res.* **89**, 5929-5952 (1984).
19. Zhou, H. *Geophys. Res. Lett.* **15**, 1425-1428 (1988).
20. Lay, T. & Young, C. *J. Geophys. Res. Lett.* **16**, 605-608 (1989).
21. Schwartz, S. Y., Lay, T. & Grand, S. *Geophys. Res. Lett.* **18**, 1265-1268 (1991).
22. Zhou, H., Anderson, D. L. & Clayton, R. W. *J. geophys. Res.* **95**, 6799-6827 (1990).
23. Aki, K., Christoffersson, A. & Husebye, E. S. *J. geophys. Res.* **82**, 277-296 (1977).
24. Kennett, B. & Engdahl, E. R. *Geophys. J.* **105**, 429-465 (1991).
25. van der Hilst, R. D. & Engdahl, E. R. *Geophys. J.* **106**, 169-188 (1991).
26. Spakman, W., Stein, S., van der Hilst, R. D. & Wortel, R. *Geophys. Res. Lett.* **16**, 1097-1101 (1989).
27. Engdahl, E. R. & Billington, S. *Bull. seismol. Soc. Am.* **76**, 77-93 (1986).
28. Engdahl, E. R. & Gubbins, D. *J. geophys. Res.* **92**, 13855-13862 (1987).
29. Jeffreys, H. & Bullen, K. E. *British Association for the Advancement of Science* (London, 1967).
30. van der Hilst, R. D. & Spakman, W. *Geophys. Res. Lett.* **16**, 1093-1096 (1989).
31. Dziewonski, A. & Anderson, D. L. *J. geophys. Res.* **88**, 3295-3314 (1983).
32. Hirahara, K. & Mikumo, T. *Phys. Earth planet Inter.* **21**, 109-119 (1980).
33. Zhou, H. & Clayton, R. W. *J. geophys. Res.* **95**, 6829-6851 (1990).
34. Kamiya, S., Miyatake, T. & Hirahara, K. *Geophys. Res. Lett.* **15**, 828-831 (1988).
35. Fukao, Y., Nenbai, M., Obayashi, M. & Inoue, H. *J. geophys. Res.* (submitted).
36. Spakman, W. & Nolet, G. in *Mathematical Geophysics* (eds Vlaar, N. J. *et al.*) 155-188 (Reidel, Dordrecht, 1988).
37. Paige, C. C. & Saunders, M. A. *ACM Trans. Math. Soft.* **8**, 3-71; 195-209 (1982).
38. Nolet, G. *J. comput. Phys.* **61**, 463-482 (1985).
39. van der Sluis, A. & van der Vorst, H. A. in *Seismic Tomography* (ed. Nolet, G.) 49-84 (Reidel, Dordrecht, 1987).
40. Okino, K., Ando, M., Kaneshima, S. & Hirahara, K. *Geophys. Res. Lett.* **16**, 1059-1063 (1989).
41. Ekström, G., Dziewonski, A. & Ibañez, J. *Eos* **71**, 1462 (1990).
42. Shearer, P. *Nature* **344**, 121-126 (1990).
43. Anderson, D. L. *Eos* **71**, 1473 (1990).
44. Humphreys, E. & Clayton, R. W. *J. geophys. Res.* **93**, 1073-1085 (1988).
45. Toy, K. thesis, Univ. of California, San Diego (1990).
46. Gurnis, M. & Hager, B. *Nature* **335**, 317-321 (1988).
47. Ringwood, A. E. & Irifune, T. *Nature* **331**, 131-136 (1988).
48. Christensen, U. & Yuen, D. A. *J. geophys. Res.* **89**, 4389-4402 (1984).
49. Machetel, P. & Weber, P. *Nature* **350**, 55-57 (1991).
50. Nataf, H. C., Moreno, S. & Cardin, P. *J. Phys. France* **49**, 1707-1714 (1989).
51. Kincaid, C. & Olson, P. *J. geophys. Res.* **92**, 13832-13840 (1987).
52. Zielhuis, A., Spakman, W. & Nolet, G. in *Digital Seismology and Modelling of the Lithosphere* (eds Cassinis, R., Panza, G. & Nolet, G.) 333-340 (Plenum, London, 1988).

ACKNOWLEDGEMENTS. We thank Brian Kennett for making the iasp models for P and S wave velocity available to us during their development. A review by Thorne Lay greatly improved the manuscript. This research was financially supported by NATO. Tomographic inversions were performed on the C205 supercomputer of the SARA (Amsterdam, The Netherlands) with financial support from the Netherlands Organization for the Advancement of Pure Research.

Assignment of G-protein subtypes to specific receptors inducing inhibition of calcium currents

C. Kleuss*, J. Hescheler†, C. Ewel*, W. Rosenthal†, G. Schultz† & B. Wittig*

* Institut für Molekularbiologie und Biochemie, and † Institut für Pharmakologie, Freie Universität Berlin, Arnimallee 22, D-W1000 Berlin 33, Germany

The inhibition of voltage-dependent Ca^{2+} channels in secretory cells by plasma membrane receptors is mediated by pertussis toxin-sensitive G proteins. Multiple forms of G proteins have been described, differing principally in their α subunits, but it has not been possible to establish which G-protein subtype mediates inhibition by a specific receptor. By intranuclear injection of antisense oligonucleotides into rat pituitary GH_3 cells, the essential role of the G_o-type G proteins in Ca^{2+}-channel inhibition is established: the subtypes G_{o1} and G_{o2} mediate inhibition through the muscarinic and somatostatin receptors, respectively.

REGULATORY GTP-binding proteins (G proteins) constitute a family of proteins involved in signal transduction across the plasma membrane[1,2]. They consist of three different subunits, α, β and γ. G proteins are classified according to their α subunits, some of which are substrates for ADP-ribosylating bacterial exotoxins, such as cholera and pertussis toxin. Sixteen G protein α subunits encoded by different genes are known, and this diversity of α subunits is increased by alternative splicing[2]. In combination with a particular α subunit, at least three types of β and four types of γ subunits contribute to an even greater variety of heterotrimeric G proteins.

G proteins transduce signals from membrane receptors to effectors like enzymes (for example, adenylyl cyclase and phospholipase C) and ion channels (such as the voltage-sensitive calcium or potassium channels). But unequivocal assignment of one G protein to a single receptor/effector system has been achieved only for G_s, the stimulatory G protein of adenylyl cyclase, and for the transducins, the retinal G proteins, which mediate between activated photoreceptors and a cyclic GMP phosphodiesterase. As the α subunits are very similar, it is difficult to distinguish between the G-protein subtypes: recombinant G-protein subunits expressed in bacteria are not particularly active, possibly because of inadequate post-translational modification[3,4], and antibodies raised against peptides corresponding to specific regions of G-protein subtypes have little affinity for native G proteins.

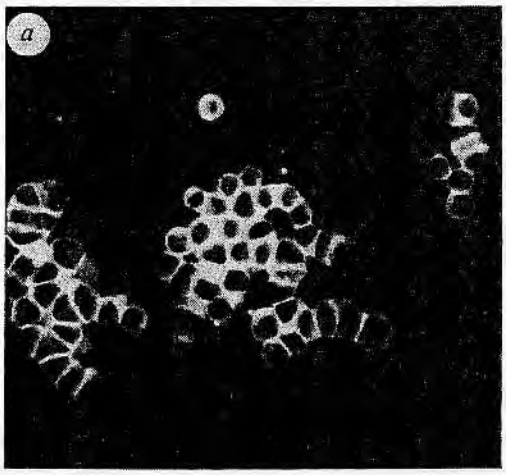
Our study concerns the G_o family, which is limited to neuronal, neuroendocrine and endocrine cells[5-7], and makes use of the fact that the distinction between the α subunits of G_o subtypes is greater at nucleic acid level than in the translated protein. We have identified selective base sequences in each α subunit and designed oligodeoxynucleotides in an antisense orientation to the respective messenger RNAs for specific 'knock-out' experiments. We microinjected these oligonucleotides directly into nuclei, the presumed site of action, in controlled amounts without significantly disturbing cell-surface functions, which might happen should they be added in high concentrations to the culture medium.

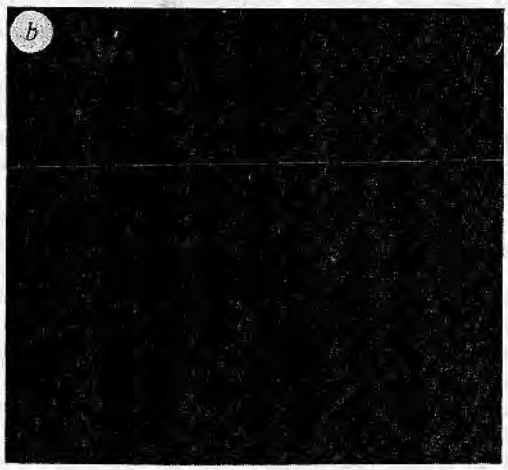
Using this approach in rat pituitary GH_3 cells, we assign a function to G_o in the receptor-induced inhibition of voltage-sensitive Ca^{2+} channels and show that the subtype G_{o1} specifically mediates the action of carbachol via a muscarinic receptor and that G_{o2} transduces the signal from the somatostatin receptor. As the cytosolic Ca^{2+} concentration is regulated by Ca^{2+}-channel activity, the receptor-induced inhibition of Ca^{2+} channels by somatostatin or carbachol could be crucial for the control of secretion.

Effectiveness of microinjected antisense DNA

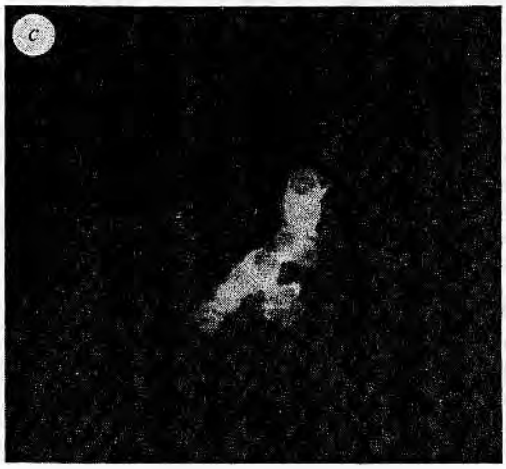
To check whether our procedure does suppress G-protein α subunits, we monitored cells by immunofluorescence microscopy following microinjection of antisense DNA. GH_3 cells express α subunits of $G_s(\alpha_s)$ and two forms each of α subunits of G_i and G_o (refs 8, 9). G-protein α subunits were

visualized by an affinity-purified α_{common}-peptide antibody[10] which recognizes α_s, α_i, α_o, α_z and the α subunit of transducin[8,11,12]. In non-injected cells, α subunits are associated mainly with the plasma membrane, as indicated by the ring-shaped pattern of fluorescence obtained with the α_{common}-peptide antibody (Fig. 1). We found that α subunits were not detectable one day after injection of the antisense oligonucleotide α-com, which corresponds to the α_{common}-peptide, indicating that expression of G-protein α subunits was reduced or even abolished. On the third day after injection, expression of α subunits had recovered. Similar results were obtained with cells injected with an α_o-specific oligonucleotide after staining with an α_o-specific antibody (data not shown).





α-com 24h



α-com 72h

G proteins in Ca^{2+} current inhibition

To show that G-protein α subunits are involved in the agonist-induced inhibition of Ca^{2+} currents in rat pituitary GH_3 cells, we made electrophysiological observations after injection of an antisense oligonucleotide common to all α subunits.

Ca^{2+} currents in GH_3 cells are probably due to activation of L-type channels[12] and are inhibited to 70–75% of control currents by carbachol and somatostatin (Fig. 2*a*) in a non-additive manner. Inhibition is abolished by treating cells with pertussis toxin. As shown previously[8,12] these Ca^{2+} currents and their inhibition are not affected by intracellularly applied cyclic AMP. It follows that G_i-mediated inhibition of adenylyl cyclase is not involved.

After injection of the oligonucleotide α-com (Fig. 2*b*), inhibition of Ca^{2+} currents by somatostatin is reduced in a time-dependent way: after 48 h, the peptide hormone is without effect, but after about 72 h the response of Ca^{2+} currents to somatostatin is restored. A similar time course of somatostatin-induced Ca^{2+} current inhibition is observed when cells are injected with the antisense oligonucleotide GS-o, which is complementary to α_o mRNAs (Fig. 2*b*). As with somatostatin, the carbachol-induced inhibition of Ca^{2+} currents is transiently abolished by each oligonucleotide (results not shown). These results show that the expression of G-protein α subunits, probably α_o, correlates with the ability of receptor agonists to inhibit Ca^{2+} currents.

The involvement of the β subunit in G-protein-mediated Ca^{2+}-current inhibition was studied by microinjecting the oligonucleotide anti-β, which is complementary to the 5′ coding regions of β_1 and β_2 mRNAs. It contains 6 nucleotides out of 29 that do not match β_3 mRNA, the longest continuous match being 13 bases. Thus, the anti-β oligonucleotide will probably form stable hybrids with β_3 mRNA (refs 13, 14). Injection of about 10,000 full-length molecules of anti-β oligonucleotide had no major influence on the receptor-induced Ca^{2+}-channel inhibition (Fig. 3). This is in contrast with the same number being fully effective in the case of antisense oligonucleotides against α subunits. If ~50,000 full-length anti-β oligonucleotide molecules are injected, inhibition of Ca^{2+} currents by somatostatin (Fig. 3) and carbachol (not shown) is abolished. The

FIG. 1 Immunocytochemical detection of G-protein α subunits in GH_3 cells injected with antisense oligonucleotides. *a*, Non-injected cells; *b* and *c*, cells intranuclearly injected with oligonucleotide α-com and incubated in culture medium for 24 h (*b*) or 72 h (*c*). Sequence of injected oligonucleotide α-com: TCATYTGCTTCACAATGGTRCTYTTYCCRGATTC, corresponding to nucleotides 133–160 of the identical strand of the α_{o2} gene sequence[39]; abbreviations for wobbled positions: R (G or A), Y (T or C), M (A or C), K (G or T), S (G or C), W (A or T)[40]. Oligonucleotide α-com can hybridize with mRNAs of all known variants of α_i, α_o, α_s and with the α subunits of transducin.
METHODS. GH_3 cells were cultured for 5 to 9 days on glass slides imprinted with numbered squares for convenient localization of the cells injected[8,12,41]. Sequences of selective oligonucleotides were detected with the help of sequence comparison and multiple alignment of the MacMolly program (Soft Gene GmbH, Berlin). Oligonucleotides were synthesized on a DNA synthesizer (Milligen Model 8600) or purchased (TIB Molbiol, Berlin). After cleavage of the protecting groups, DNA was extracted with phenol and chloroform and precipitated with ethanol. Intranuclear injections of oligonucleotides (5 μM) were performed with an automated injection system (Zeiss). No difference in effectiveness of injected oligonucleotides could be detected if they were dissolved in a physiological nuclear buffer ('new' buffer[42]) instead of water. About 3,000 cells were injected by means of commercial microcapillaries (Femtotips, Eppendorf) with an outlet diameter of 0.2 μm. Injection time was 0.1 s, the pressure 20–30 hPa. The calculated injected volume was 10–20 fl (ref. 43) containing 10,000 to 20,000 full-length oligonucleotides. Indirect immunofluorescence staining was carried out as described[44]. After fixation cells were incubated with the first antibody overnight at 6–8 °C. The affinity-purified α_{common}-peptide antibody, which recognize the α subunits of α_s, α_{i1}, α_{i2}, α_{i3}, α_{o1}, α_{o2}, α_z and transducin, was diluted 100-fold in phosphate-buffered saline. Incubation with the second antibody, 1:100 diluted goat anti-rabbit IgG-fluoroscein isothiocyanate conjugate (Sigma), was for 1 h at room temperature. Scale bar, 20 μm.

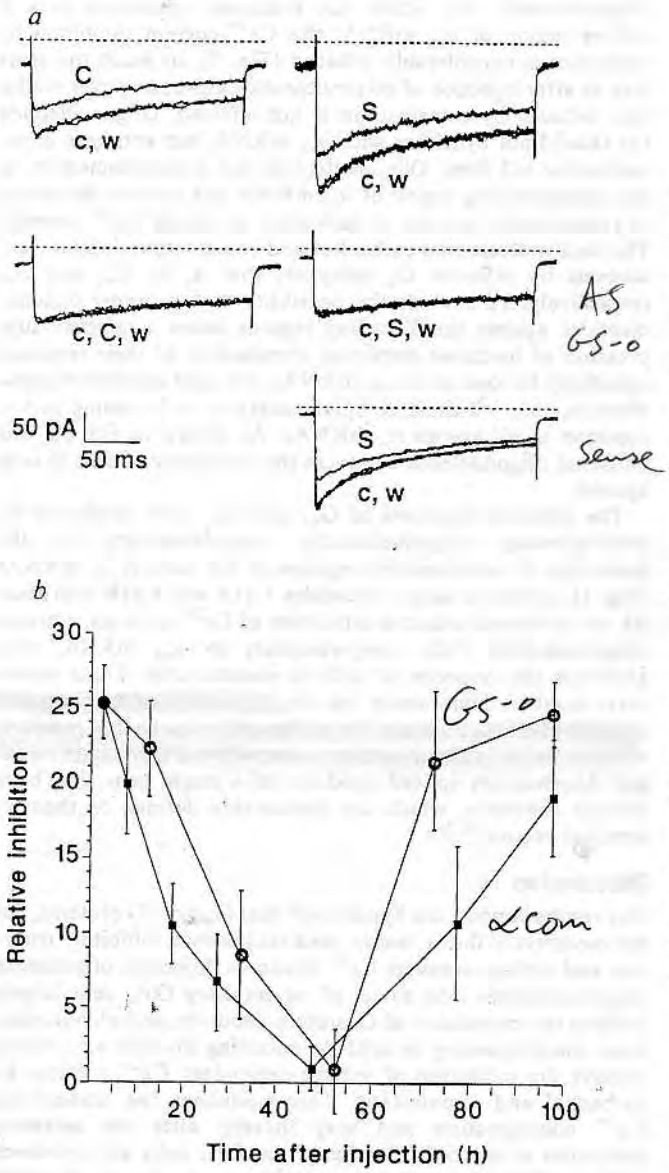


FIG. 2 *a*, Ca^{2+}- current inhibition by somatostatin and carbachol in GH_3 cells injected with antisense oligonucleotides. Whole-cell Ca^{2+} currents were recorded under voltage-clamp conditions by depolarizing pulses from −80 to 0 mV. c, Control currents before application of receptor agonist; S, currents during superfusion of cells with 1 μM somatostatin; C, currents during superfusion of cells with 10 μM carbachol; w, currents after removal of receptor agonists. Top: non-injected cell; middle: cell injected with GS-o; bottom: cell injected with sense-α. *b*, Time course of Ca^{2+}-current inhibition by somatstatin (1 μM) in GH_3 cells injected with antisense oligonucleotides. Oligonucleotides α-com (filled squares) and GS-o (open circles) were injected at time zero. Mean values are shown ($n \geq 10$). Sequences of injected oligonucleotides: GS-o, CGCCTTGCYCCGCTCGAG, corresponding to nucleotides 60–78 of the identical strand of the α_{o2} gene sequence[39]. GS-o can hybridize with mRNAs of all known variants of α_o; α-com, see legend to Fig. 1; sense-α, GAATCYGGRAARAGYACCATTGTGAAGCARATGA, corresponding to nucleotides 133–160 of the identical strand of the α_{o2} gene sequence[39]. It has reverse complementarity to α-com. For abbreviations denoting wobbled positions, see legend to Fig. 1.

METHODS. Cells were grown on coverslips until they reached a density of 500–1,000 cells per frame (1 × 1 mm^2). Oligonucleotides were injected into all cells within one frame. For determination of Ca^{2+} currents, coverslips were transferred to a chamber (0.2 ml) mounted on an inverted microscope. The chamber was continuously perfused with a solution with Ba^{2+} as charge carrier, containing 125 mM NaCl, 10.8 mM $BaCl_2$, 1 mM $MgCl_2$, 5.4 mM CsCl, 10 mM glucose and 10 mM Na-HEPES (pH 7.4 at 37 °C); flow rate was 5 ml min^{-1}. The pipette solution contained 120 mM CsCl, 1 mM $MgCl_2$, 3 mM Mg-ATP, 10 mM EGTA, 10 mM Na-HEPES (pH 7.4). Whole-cell membrane currents were measured according to ref. 45 using a List/EPC7 patch-clamp amplifier. The holding potential was −80 mV. Inward currents were recorded during 200-ms long test potentials to 0 mV; stimulation rate was 0.5 Hz. The shape, amplitude and inactivation characteristics of Ca^{2+} currents were not affected by microinjection itself. In some cells a rapid inactivating component was eliminated by those antisense oligonucleotides that suppress the agonist-induced inactivation of the voltage-sensitive Ca^{2+} current.

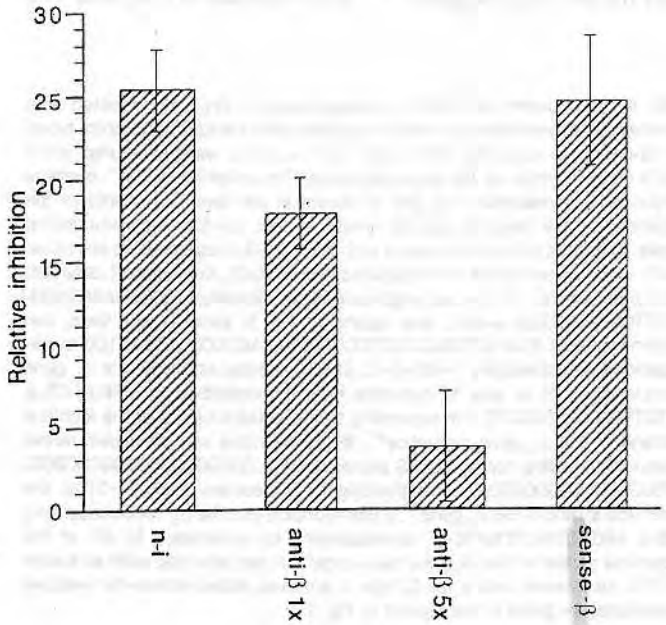


FIG. 3 Ca^{2+}-current inhibition by somatostatin in GH_3 cells injected with antisense oligonucleotides directed against mRNAs encoding β subunits. Whole-cell Ca^{2+} currents were measured 24 h after injection of the oligonucleotide indicated. The inhibition of Ca^{2+} currents by somatostatin (1 μM) is shown in per cent of currents observed in the absence of the receptor agonist (mean values are given; $n \geq 8$). n-i, Non-injected cells; anti-β 1× and anti-β 5×, about 10,000 and 50,000 full-length molecules respectively, were injected per nucleus. Cells that had been injected with anti-β 1× responded to somatostatin in the same way as non-injected cells ($P < 0.01$, Kolmogoroff–Smirnoff test). Sequences of injected oligonucleotides: anti-β, GTSCKCATYTGGATTCTYCCCACWGGGTC, corresponding to nucleotides 112–140 of the identical strand of the β_1 gene sequence[46]. It can hybridize with the three known sequences of β mRNAs (see text); sense-β, GACCCW-GTGGGRAGAATCCARATGMGSAC, corresponding to nucleotides 112–140 of the identical strand of the β_1 gene sequence[46]. It has reverse complementarity to anti-β. Abbreviations for wobbled positions are given in the legend to Fig. 1.

effects of anti-β oligonucleotides are specific, because the corresponding sense oligonucleotide, sense-β, has no effect. Our results emphasize the requirement for β subunits in the interaction between receptor and G protein.

G_o inhibits Ca^{2+} currents

To determine whether the loss of Ca^{2+}-current inhibition is due to the suppression of α_o, we tested the ability of various antisense oligonucleotides to interfere with the receptor-induced inhibition of Ca^{2+} currents. As we have already shown, oligonucleotide GS-o, which is complementary to the translated 5′-terminal regions of α_o mRNAs, largely reduces the somatostatin-induced inhibition of Ca^{2+} currents (Fig. 4). In contrast, the antisense oligonucleotides complementary to translated 5′-terminal regions of α_s and α_i mRNAs, GS-s and GS-i respectively, have no effect. The sense oligonucleotide (sense-α) corresponding to α-com, or an oligonucleotide with an unrelated sequence (nonsense) are likewise without effect. Similar results are obtained if carbachol is used instead of somatostatin (data not shown).

We also injected antisense oligonucleotides complementary to sequences located immediately upstream from the translation start as the diversity of α-subunit mRNAs is more pronounced in the leader sequence than in the coding regions. This diversity permitted the use of long oligonucleotides (>20 nucleotides) which form stronger hybrids with the target sequence and should retain the ability to hybridize even after removal of bases by nuclear exonucleases. As shown in Fig. 4, the effects of antisense oligonucleotides that can hybridize with 5′-untranslated regions of α_s mRNA (5′s2) and α_o mRNA (5′o3) are simlar to the those of oligonucleotides that hybridize with the translated regions of the corresponding mRNAs.

Our data confirm that G_o is involved in the receptor-induced inhibition of voltage-sensitive Ca^{2+} channels. This hypothesis was based on two observations made in neuronal cells: (1) the ability of purified α_o to restore the receptor-induced inhibition of Ca^{2+} currents in pertussis toxin-treated cells[6,15–17], and (2) the attenuation of the inhibitory modulation of Ca^{2+} currents by α_o antibodies[16,18].

Differential functions of G_o subtypes

Alternative splicing of a single transcript encoding α_o subunits[19] yields three mRNAs, α_{o1A}, α_{o1B}, and α_{o2} (refs 20, 21). As α_{o1A} and α_{o1B} mRNAs differ only in the 3′ noncoding regions, they encode the same protein, namely the α_{o1} subunit. The α_{o2} mRNA encodes the α_{o2} subunit. The two α_o subunits differ only in their C-terminal regions, which are assumed to be the recognition site for activated receptors[22,23]. After injection of GH_3 cells with oligonucleotide to1, which has antisense orientation to a 3′ coding region of α_{o1} mRNA, the Ca^{2+}-current inhibition by carbachol is considerably reduced (Fig. 5), in much the same way as after injection of oligonucleotide GS-o; however inhibition induced by somatostatin is not affected. Oligonucleotide to1 should not hybridize with α_{o2} mRNA, but antisense oligonucleotide to2 does. Oligonucleotide to2 is complementary to the corresponding region of α_{o2} mRNA and reduces the ability of somatostatin but not of carbachol to inhibit Ca^{2+} currents. The data indicate that carbachol and somatostatin inhibit Ca^{2+} currents by different G_o subtypes, that is, by G_{o1} and G_{o2} respectively. To exclude the possiblity that antisense oligonucleotides against the 3′ coding regions cause a selective suppression of hormone responses irrespective of their sequence specificity for one of the α_o mRNAs, we used another oligonucleotide, to3, which is complementary to a 3′ coding region common to all known α_o mRNAs. As shown in Fig. 5*a*, this antisense oligonucleotide reduces the sensitivity of cells to each agonist.

The different functions of G_{o1} and G_{o2} were confirmed by microinjecting oligonucleotides complementary to the individual 3′ nontranslated regions of the various α_o mRNAs (Fig. 5). Antisense oligonucleotides 3′o1A and 3′o1B both abolish the carbachol-induced inhibition of Ca^{2+} currents, whereas oligonucleotide 3′o2, complementary to α_{o2} mRNA, only abolishes the response of cells to somatostatin. These results taken together demonstrate that the α_{o1} subunit of the G-protein trimeric complex mediates the action of the carbachol receptor, whereas the α_{o2} subunit mediates that of the somatostatin receptor. Alternatively spliced products of a single gene thus have distinct functions, which are presumably defined by their C-terminal regions[22,23].

Discussion

Our results support the hypothesis[6] that G_o-type G proteins, but not members of the G_i family, mediate between inhibitory receptors and voltage-sensitive Ca^{2+} channels. Injection of antisense oligonucleotides into nuclei of rat pituitary GH_3 cells largely inhibits the expression of G-protein subunits, and oligonucleotides complementary to mRNAs encoding the two α_o subunits remove the inhibition of voltage-dependent Ca^{2+} currents by carbachol and somatostatin. This modulates the intracellular Ca^{2+} concentration and may thereby alter the secretion behaviour of the cell. Our data from GH_3 cells are consistent with an effect observed in rat pituitary tumour cells, in which expression of α_o was correlated with the inhibition of prolactin secretion by dopamine[24]. Oligonucleotides complementary to

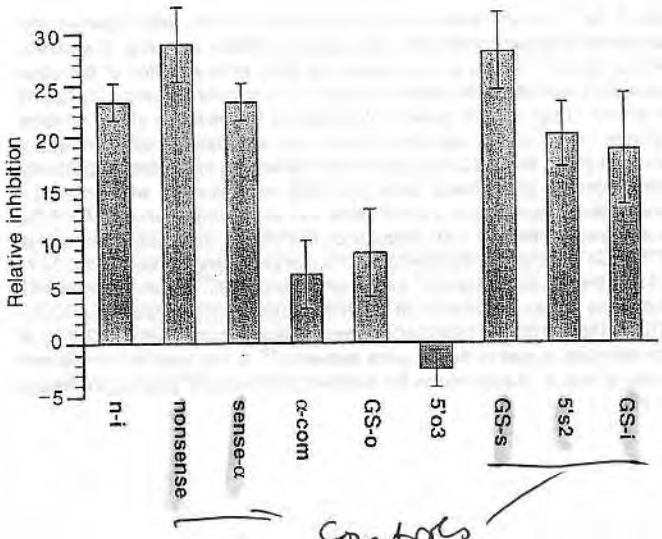


FIG. 4 Ca^{2+}-current inhibition by somatostatin in GH_3 cells injected with antisense oligonucleotides directed against mRNAs encoding different types of G-protein α subunits. Whole-cell Ca^{2+} currents were measured about 24 h after injection of the oligonucleotide. The inhibition of Ca^{2+} currents induced by somatostatin (1 μM) is shown in per cent of currents in the absence of the receptor agonist (mean values; $n \geq 10$). n-i, Non-injected cells. Cells that had been injected with 5′s2 or GS-i responded to somatostatin in the same way as non-injected cells ($P < 0.01$, Kolmogoroff–Smirnoff test). Sequences of injected oligonucleotides: nonsense, GGGGGAAGTAGGTCTTGGTGGTGGGG; α-com, see legend to Fig. 1; sense-α and Gs-o, see legend to Fig. 2: 5′o3, GGTGGCCCCTTCCCTGCCACAGCCCGCACGACTCG, corresponding to nucleotides (−35)–(−1) of the identical strand of the α_o gene sequence [47]. It is able to hybridize with the described α_o mRNA; GS-s, TTGTTGGCCTCRCGCTG, corresponding to nucleotides 54–70 of the identical strand of the α_s gene sequence[47]. It can hybridize with all known mRNA sequences coding for G_s-type G proteins; 5′s2, GGCGGCGGCGGGGCGCGGCCGGGCTGCGGGGCGGCG, corresponding to nucleotides (−36)–(−1) of the identical strand of the α_s gene[47]. It can hybridize with the α_s mRNA described; GS-i, ARGTTSYKGTCGATCAT, corresponding to nucleotides 51–67 of the identical strand of the α_i gene sequence[47]. It can hybridize with all known mRNA sequences coding for G_i-type G proteins. Abbreviations for wobbled positions are given in the legend to Fig. 1.

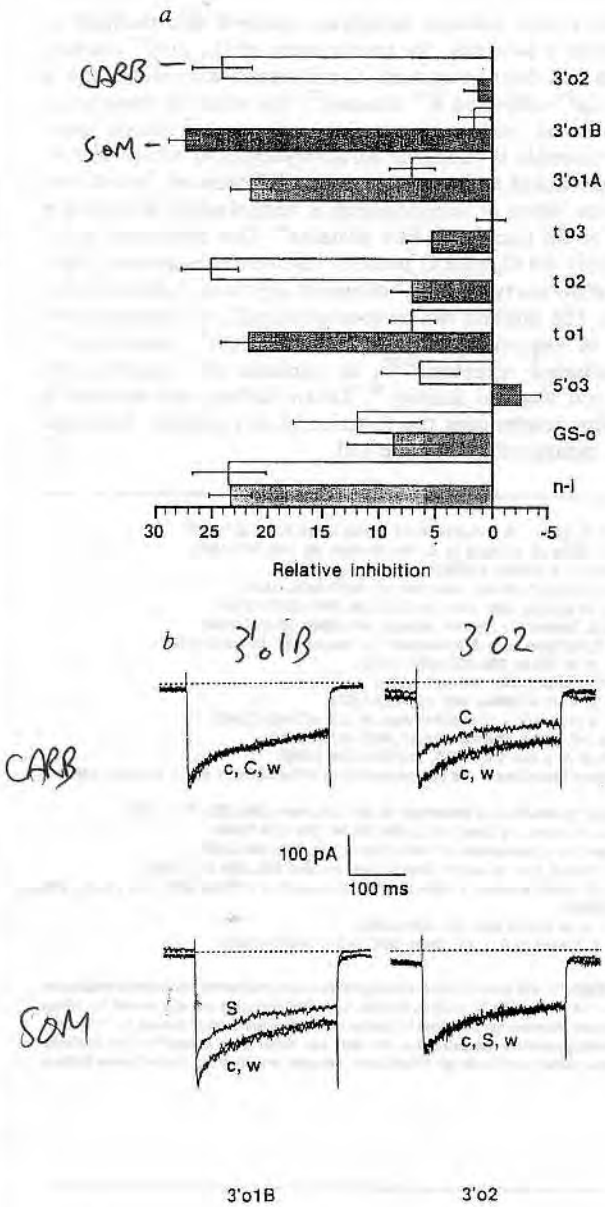


FIG. 5 Ca^{2+} current inhibition by hormones in GH_3 cells injected with antisense oligonucleotides directed against mRNAs encoding α_o. Whole-cell Ca^{2+} currents were measured about 24 h after injection of the oligonucleotide. *a*, Inhibition of Ca^{2+} currents by somatostatin (1 μM; shaded bars) or carbachol (10 μM; open bars) as a per cent of the currents observed in the absence of the respective agonist (mean values; $n \geq 10$). n-i, Non-injected cells. *b*, Time-current recordings of the voltage-sensitive Ca^{2+} channel in GH_3 cells in the presence of carbachol (upper) or somatostatin (lower). Left, one cell injected with 3'o1B; right, one cell injected with 3'o2. See legend of Fig. 2 for abbreviations. Sequences of injected oligonucleotides: nonsense, see legend to Fig. 4; GS-o, see legend to Fig. 2; 5'o3, see legend to Fig. 4; to1, AGGCAGCTGCATCTTCATAGGTGTT, corresponding to nucleotides 907–930 of the identical strand of the α_{o1} gene sequence[39]. It can hybridize with α_{o1} mRNA; to2, GAGCCACAGCTTCTGTGAAGGCACT, corresponding to nucleotides 907–930 of the identical strand of the α_{o2} gene sequence[39]. It can hybridize with α_{o2} mRNA; to3, TGGIGCCTGIGTATTCAGG, corresponding to nucleotides 888–906 of the identical strand of the α_{o1}/α_{o2} gene sequence[21]. It can hybridize with both α_o mRNAs; 3'o1A, TCAACAGCAAAGAGTCCATGAAGCAGT, corresponding to nucleotides 1,093–1,119 of the identical strand of the '$G_oA\alpha$ clone G_o12' sequence[21]. It can hybridize with the described α_{o1A} mRNA; 3'o1B, CAAGCTGTTCTGTGAGTGCTGGAAT, corresponding to nucleotides 1,096–1,120 of the identical of the '$G_oA\alpha$ clone G_o11' sequence[21]. It can hybridize with the described α_{o1B} mRNA; 3'o2, CAGAGCTCTGGGTCCAGGGGAGAAGTG, corresponding to nucleotides 1,093–1,119 of the identical strand of the α_{o2} gene sequence[21]. It can hybridize with α_{o2} mRNA. Abbreviations for wobbled positions are given in the legend to Fig. 1.

α_{o1} mRNA selectively interfered with Ca^{2+}-current inhibition by carbachol, whereas oligonucleotides complementary to α_{o2} mRNA specifically abolished the somatostatin-induced inhibition of Ca^{2+} currents. Intranuclear injection of oligonucleotides complementary to the mRNAs encoding G-protein β subunits also prevented Ca^{2+}-current inhibition. This demonstrates the absolute requirement of β subunits for effective coupling between receptor and G protein.

The successful repression of G protein function implies that the inhibition of synthesis lasts longer than the lifetime of subunits already expressed. The half-lives of α_o subunits range from 21 h to 35 h under steady-state conditions[25]. In our experiments, immunofluorescence of G-protein α subunits was barely detectable 24 h after injection of oligonucleotides (Fig. 1). Function was impaired 19 h after injection of oligonucleotides (Fig. 2). This discrepancy can be explained by the disturbance of gene regulation or of steady-state levels by the injection of oligonucleotides, leading to an altered lifetime of G-protein subunits.

The selective repression of individual genes by nucleic acids in antisense orientation, that is, complementary to their mRNAs, has often been demonstrated[26] (for review, see refs 27, 28). If antisense DNA oligonucleotides are delivered to recipient cells, they hybridize to complementary regions in mRNA and render it succeptible to cellular RNase activity[29,30].

Conventional techniques for delivering antisense nucleic acids into mammalian cells are not suitable for suppressing G protein α subunits: only a few short sequence regions allow for the selective hybridization of antisense oligonucleotides. Such short regions cannot be transcribed either transiently or permanently into antisense RNA from recombinant eukaryotic expression vectors. Alternatively, short DNA oligonucleotides can be delivered to the cytoplasm of mammalian cells by addition to the culture medium at high concentration[27]. But the efficiency of uptake and the amount of oligonucleotide surviving passage through degradative organelles is influced by the culture medium, the cell type, the stage of the cell cycle, the length of oligonucleotide and probably its sequence[31]. Moreover, incubating cells at micromolar concentrations of an oligonucleotide might interfere with cell-surface functions. Taking these considerations into account, we delivered our DNA oligonucleotides into nuclei by microinjection, a technique virtually independent of oligonucleotide length and sequence: the oligonucleotides are applied to the presumed site of action, the amount reaching the nucleus is controlled, and the optimal culture environment is unchanged.

Although we do not consider here the mechanism of antisense repression of genes (for review, see ref. 32), our results might help to specify the target level. A translational block due to a hybridization complex between antisense DNA oligonucleotide and mRNA is very unlikely to be involved. Depending on the mRNA region, such a complex could either interfere with translation initiation or lead to premature termination as a result of stalling by ribosomes. Our data show that the repression is independent of assumed hybridization positions. Oligonucleotides against untranslated sequences upstream or downstream from coding sequences are equally effective in repressing α subunits. In particular, antisense oligonucleotides hybridizing in 3′ untranslated regions of α_o mRNA (3'o1A, 3'o1B, 3'o2) cannot cause premature termination.

Considering quantitative conditions, we routinely injected $\sim 10^4$ antisense oligonucleotide molecules into each nucleus: the same degree of repression was found with 10^3 molecules (results not shown), but when 10^2 molecules were injected per nucleus, the repression disappeared. Assuming a steady-state amount of 10 molecules of α_o mRNA per GH_3 cell, an initial 100-fold excess of oligonucleotide over mRNA is sufficient to reduce expression. After intranuclear injection, the number of free oligonucleotide molecules per cell will be reduced by cell divisions, degradation by single strand-specific DNA

exonucleases and by the turnover of the mRNA population. The very low number of molecules necessary for repression can be explained if the hybridization structure inhibits the chromatin template function or if antisense oligonucleotides are continuously re-used after degradation of transcripts. The selective repression of splice variants α_{o1} and α_{o2} argues against the former mechanism. Thus continuous cycles of hybrid formation, the cutting of transcripts at the hybrid site by RNase H, the liberation of antisense oligonucleotide, and hybridization with another α_o transcript are the most likely mechanisms[13]. If the concentration of free antisense oligonucleotide is low and hybridization complexes are the predominant structures, oligonucleotides are protected from degradation.

Antisense oligonucleotides can be used to study the role of any G-protein subunit: for instance, somatostatin and carbachol not only inhibit Ca^{2+} currents but also stimulate a 55 pS-K^+ channel in GH_3 cells[22]. On the basis of reconstitution experiments with isolated membrane patches and purified or recombinant α subunits, the involvement of G_{i3} in K^+-channel regulation has been proposed. Somatostatin also stimulates a 120 pS-, Ca^{2+}-activated K^+ channel[33]; this effect is sensitive to pertussis toxin and may require a dephoshorylation step. Another example is found in atrial myocytes in which acetylcholine stimulates an inward rectifying K^+ channel; in cell-free systems, the effect of acetylcholine is mimicked by activated α subunits of all known G_i-like proteins[34]. Our approach could help identify the G_i-type G proteins involved in hormonal regulation of different types of K^+ channels in pituitary cells or atrial myocytes. The method can be used principally to determine the function of sequenced Ca^{2+}-channel subunits[35], numerous G protein-coupled receptors[36,37], or subunits of receptor with intrinsic ion channel activity[38]. Taken further, the method is suitable for determining the function of any protein, provided it can be measured in a single cell. □

Received 17 June; accepted 8 August 1991.

1. Birnbaumer, L. *A. Rev. Pharmac. Toxicol.* **30**, 675-705 (1990).
2. Simon, M. I., Strathmann, M. P. & Gautam, N. *Science* **252**, 802-808 (1991).
3. Graziano, M. P., Freissmuth, M. & Gilman, A. G. *J. biol. Chem.* **264**, 409-418 (1989).
4. Mattera, *et al. J. biol. Chem.* **264**, 465-471 (1989).
5. Asano, T., Semba, R., Kamiya, N., Ogasawara, N. & Kato, K. *J. Neurochem.* **50**, 1164-1169 (1988).
6. Hescheler, J., Rosenthal, W., Trautwein, W. & Schultz, G. *Nature* **325**, 445-447 (1987).
7. Schultz, G., Rosenthal, W., Trautwein, W. & Hescheler, J. *A. Rev. Physiol.* **52**, 275-292 (1990).
8. Offermanns, S. *et al. Molec. Endocr.* **5**, 995-1002 (1991).
9. Paulssen, E. J., Paulssen, R. H., Haugen, T. B., Gautvik, K. M. & Gordeladze, J. O. *Molec. Cell. Endocr.* **76**, 45-53 (1991).
10. Mumby, S. M., Kahn, R. A., Manning, D. R. & Gilman, A. G. *Proc. natn. Acad. Sci. U.S.A.* **83**, 265-269 (1986).
11. Offermanns, S., Schultz, G. & Rosenthal, W. *J. biol. Chem.* **266**, 3365-3368 (1991).
12. Rosenthal, W. *et al. EMBO J.* **7**, 1627-1633 (1988).
13. Cazenave, C. *et al. Nucleic Acids Res.* **17**, 4255-4272 (1989).
14. Zamecnik, P. C. & Stephenson, M. L. *Proc. natn. Acad. Sci. U.S.A.* **75**, 280-284 (1978).
15. Ewald, D. A., Sternweis, P. C. & Miller, R. J. *Proc. natn. Acad. Sci. U.S.A.* **85**, 3633-3637 (1988).
16. Harris-Warrick, R. M. *et al. Neuron* **1**, 27-32 (1988).
17. Toselli, M., Lang, J., Costa, T. & Lux, H. D. *Pflügers Arch. ges. Physiol.* **415**, 255-261 (1989).
18. McFadzean, I., Mullaney, I., Brown, D. A. & Milligan, G. *Neuron* **3**, 177-182 (1989).
19. Tsukamoto, T. *et al. Proc. natn. Acad. Sci. U.S.A.* **88**, 2974-2978 (1991).
20. Bertrand, P., Sanford, J., Rudolph, U., Codina, J. & Birnbaumer, L. *J. biol. Chem.* **265**, 18576-18580 (1990).
21. Strathmann, M., Wilke, T. M. & Simon, M. I. *Proc. natn. Acad. Sci. U.S.A.* **87**, 6477-6481 (1990).
22. Birnbaumer, L., Abramowitz, J. & Brown, A. *Biochim. biophys. Acata* **1031**, 163-224 (1990).
23. Masters, S. E., Strout, R. N. & Bourne, H. R. *Protein Engng* **1**, 47-54 (1986).
24. Collu, R. *et al. Endocrinology* **122**, 1176-1178 (1988).
25. Silbert, S., Michel, T., Lee, R. & Neer, E. J. *J. biol. Chem.* **265**, 3102-3105 (1990).
26. Izant, J. G. & Weintraub, H. *Cell* **36**, 1007-1015 (1984).
27. Weintraub, H., Izant, J. G. & Harland, R. M. *Trends Genet.* **1**, 22-25 (1985).
28. Green, P. J., Pines, O. & Inouye, M. *A. Rev. Biochem.* **55**, 569-597 (1986).
29. Shuttleworth, J. & Colman, A. *EMBO J.* **7**, 427-434 (1988).
30. Minshull, J. & Hunt, T. *Nucleic Acids Res.* **14**, 6433-6451 (1986).
31. Loke, S. L. *et al. Proc. natn. Acad. Sci. U.S.A.* **86**, 3474-3478 (1989).
32. Hélène, C. & Toulmé, J.-J. *Biochim. biophys. Acta* **1049**, 99-125 (1990).
33. White, R. E., Schonbrunn, A. & Armstrong, D. L. *Nature* **351**, 570-573 (1991).
34. Yatani, A. *et al. Nature* **336**, 680-682 (1988).
35. Mori, Y. *et al. Nature* **350**, 398-402 (1991).
36. Sunahara, R. K. *et al. Nature* **350**, 614-619 (1991).
37. Findlay, J. & Eliopoulos, E. *Trends Pharmac. Sci.* **11**, 492-499 (1990).
38. Changeux, J.-P. *Trends Pharmac. Sci.* **11**, 485-492 (1990).
39. Hsu, W. H. *et al. J. biol. Chem.* **265**, 11220-11226 (1990).
40. Nomenclature Committee of the International Union of Biochemistry *Eur. J. Biochem.* **150**, 1-5 (1985).
41. Offermanns, S., Schultz, G. & Rosenthal, W. *Eur. J. Biochem.* **180**, 283-287 (1989).
42. Jackson, D. A., Yuan, J. & Cook, P. R. *J. Cell Sci.* **90**, 365-378 (1988).
43. Graessmann, M. & Graessmann, A. *Meth. Enzym.* **101**, 482-492 (1983).
44. Dreyer, C., Scholz, E. & Hausen, P. *Roux's Arch. dev. Biol.* **191**, 228-233 (1982).
45. Hamill, O. P., Marty, A., Neher, E., Sakmann, B. & Sigworth, F. J. *Pflügers. Arch. ges. Physiol.* **391**, 85-100 (1981).
46. Tanabe, T. *et al. Nature* **315**, 242-245 (1985).
47. Jones, D. T. & Reed, R. R. *J. biol. Chem.* **262**, 14241-14249 (1987).

ACKNOWLEDGEMENTS. We thank C. Crane-Robinson (Portsmouth Polytechnic) for a critical discussion and revision of the manuscript; D. Wedlich (Institut für Molekularbiologie und Biochemie) for advice in immunochemical detection methods, and K. Spicher and K.-D. Hinsch (both of Institut für Pharmakologie) for antibody generation and purification. Our work was supported by grants from the Deutsche Forschungsgemeinschaft and Fonds der Chemischen Industrie, and from the Trude-Goerke-Stiftung (B.W.).

LETTERS TO NATURE

Morphological differences between optical and infrared images of the spiral galaxy NGC309

David L. Block*† & Richard J. Wainscoat†

* Department of Computational and Applied Mathematics, Witwatersrand University, Private Bag 3, Wits 2050, South Africa
† Institute for Astronomy, University of Hawaii, 2680 Woodlawn Drive, Honolulu, Hawaii 96822, USA

THE morphological classification of spiral galaxies into various types is suspected to be highly dependent on the wavelength of observation[1], because optical images emphasize young population I stars at the expense of other stellar types, as well as ionized gas and dust. Extension of the classification of galaxies out to wavelengths of a few micrometres has had to await the development of large-format near-infrared array cameras. We present here images at 2.1 μm wavelength of NGC309, one of the largest 'grand design' (type ScI) spiral galaxies, obtained with the 256×256 array camera developed for the NICMOS (near-infrared camera and multiobject spectrograph) instrument, designed for installation on the Hubble Space Telescope. Optically, NGC309 presents a classic multi-arm morphology, but at 2.1 μm we see a two-arm spiral and the appearance of a prominent central bar; it resembles the SBa galaxy NGC1358. These studies underscore existing indications[2] that the disk structure of spiral galaxies may be unrelated to the Hubble type assigned from the transient population I morphology.

Pioneering work[3,4] in the detection of smooth 'red' arms in the disk of the grand design spiral M51 (NGC5194) showed that the morphology of the evolved disk population need not mimic the Hubble classification assigned from the young population I tracers (OB stellar associations and ionized hydrogen regions). In 1976, Strom, Jensen and Strom[5] commented: "Detailed study of the underlying density wave in late type spirals is considerably complicated by the dominance of the bright Population I tracers near the wave crests. This problem can be avoided, in part, by photographing these galaxies at near infrared wavelengths .... However, technical difficulties have thus far precluded such observations."

Previous investigations[6-9] have been restricted to wavelengths shorter than 1 μm. Here, we report galactic structure results at 2.1 μm using a large format near-infrared array camera, for the grand-design Hubble type-c galaxy NGC309.

NGC309 is one of the most luminous spirals in the Revised