A specific G_o heterotrimer couples somatostatin receptors to voltage-gated calcium channels in RINm5F cells

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Received 12 December 1995; revised version received 29 December 1995

Abstract The peptide hormone somatostatin inhibits glucose-induced insulin secretion in the rat insulinoma RINm5F cells by inhibition of voltage-gated calcium channels. Here we used microinjection of antisense oligonucleotides directed against subtypes of G-protein subunits to determine the subunit composition involved in somatostatin-induced inhibition of voltage-gated calcium channels in RINmF5 cells. Injection of antisense oligonucleotides annealing to the respective mRNA of $G\alpha_{o2}$, $G\beta_1$ and $G\gamma_3$ reduced the somatostatin-induced inhibition of calcium channels in these cells. Injection of antisense oligonucleotides directed against other G-protein subunits did not, suggesting that in RINm5F cells the somatostatin receptor couples to a G protein of $G\alpha_{o2}\beta_1\gamma_3$ composition. By using a selective agonist of type 2 somatostatin receptors (SSTR 2) NC 8–12, we identified this receptor as the subtype coupling to calcium channels in RINm5F cells.

Key words: Somatostatin receptor; G-protein heterotrimer; RINm5F microinjection; Antisense oligonucleotide

1. Introduction

Somatostatin, a peptide hormone of 14 or 28 aminoacids, exerts many physiological effects on endocrine and nerve cells. It is the major physiological inhibitor of growth hormone release from the pituitary and of insulin and glucagon secretion from the pancreas. The intracellular effects caused by somatostatin are mediated by a family of five related receptors which are members of the superfamily of heptahelical receptors (for reviews see [1–6]). These receptors interact with heterotrimeric guanine nucleotide-binding proteins (G proteins). G proteins represent a family of proteins mediating signal transduction from the outside of the cell to the cytoplasm by interacting with receptor and effector proteins. They are composed of three subunits, α, β and γ, of which at least 23 different α forms ($G\alpha$) (including splice variants), 5 different β forms ($G\beta$) and 11 different γ forms ($G\gamma$) exist. The cellular effects mediated by somatostatin are inhibition of adenylyl cyclase, stimulation of K^+ channels and inhibition of voltage-gated Ca^{2+} channels. All effects are mediated by pertussis toxin (PTX)-sensitive G proteins of the $G_{i/o}$ family. In the insulin-secreting cell lines HIT and RINm5F, somatostatin inhibits voltage-gated Ca^{2+} channels suggesting this as one mechanism by which somatostatin inhibits glucose-induced insulin release in these cell lines [7,8]. Previously, we used microinjection of antisense oligonucleotides to identify the subunit composition of heterotrimeric G proteins mediating modulation of Ba^{2+} current through voltage-gated Ca^{2+} channels (I_{Ca}). In GH_3 cells the M_4 muscarinic receptor couples to the G protein trimer consisting of $\alpha_{o1}\beta_3\gamma_4$, the somatostatin receptor uses the trimer $\alpha_{o1}\beta_1\gamma_3$, and the galanin receptor couples to the trimers $\alpha_{o1}\beta_2\gamma_2$ and $\alpha_{o1}\beta_3\gamma_4$. The latter receptor uses the same trimers to inhibit voltage-gated Ca^{2+} channels in the insulinoma cell line RINm5F [9–12]. Here we used the same method to determine the subunit composition of the G proteins involved in inhibition of voltage-gated Ca^{2+} channels by somatostatin in RINm5F cells.

2. Materials and methods

2.1. Materials

Rat galanin was purchased from Saxon Biochemicals (Hannover, Germany), pertussis toxin was from List Biological Laboratories (Campbell, USA), somatostatin, [ethylenebis (oxyethylenenitrilo)]tetraacetic acid (EGTA) and 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid (BAPTA) were from Sigma (Deisenhofen, Germany). All other chemicals were from Sigma (Deisenhofen, Germany) or Merck (Darmstadt, Germany). Galanin and somatostatin were used at the final concentrations of 500 nM and 1 μM, respectively.

2.2. Cell culture

The insulin-secreting cell line RINm5F was obtained from the American Type Culture Collection (Rockville, USA) and was cultured using RPMI-1640 medium containing 10% fetal calf serum, 2 mM L-glutamine, 0.1 mg/ml streptomycin and 100 units/ml penicillin in an atmosphere of 5% CO_2 in air. One day prior to microinjection, trypsinized cells were seeded at a density of 200–1000 cells/mm^2 in plastic Petri dishes containing glass slides imprinted with squares for localization of injected cells. If indicated, PTX was added to the medium at a concentration of 100 ng/ml 24 h prior to experiments.

2.3. Oligonucleotides and inection

The sequences of oligonucleotides were chosen by sequence comparison and multiple alignment using MacMolly Tetra software (Soft Gene, Berlin, Germany). The base sequences of rat mRNAs are not known for all G-protein subunits studied here. Therefore, we used the statistical approach of preferred codon usage in the rat to obtain the most likely sequences. For details see Kalbrenner et al. [12] and Degtiar et al. (submitted). The oligonucleotide named anti-α_{com} is able to anneal to a nucleotide sequence common to the mRNAs of all PTX-sensitive G proteins; it is reverse-complementary to sense-α_{com}. These oligonucleotides as well as anti-α_{ocom}, anti-α_{icom}, anti-α_{o1}, anti-α_{o2}, anti-α_q, anti-α_{11} and anti-α_z are identical to those used in previous studies [9,12,13]. The oligonucleotides sense-β_3, anti-β_1, anti-β_2, anti-β_3, anti-β_4, anti-γ_1, anti-γ_2, anti-γ_3, anti-γ_4 were also used before [10–12]. Oligodesoxyribonucleotides were synthesized in a DNA synthesizer (Milligen, model 8600); chimeric phosphorothioate-phosphodiester oligodesoxyribonucleotides were synthesized using the method described by Iyer et al. [14]. Injections of oligonucleotides were performed by using a manual injection system (Eppendorf, Hamburg, Germany). The injection solution routinely contained 10 μM oligonucleotides in water; use of other concentrations (5 or 15 μM) for some experiments did not influence the results. Increases in nuclear and entire cell volumes were used as a visual control for successful injection (presumably 10–20 fl were injected). To measure microinjection efficiency, cells were injected with a 10 μM

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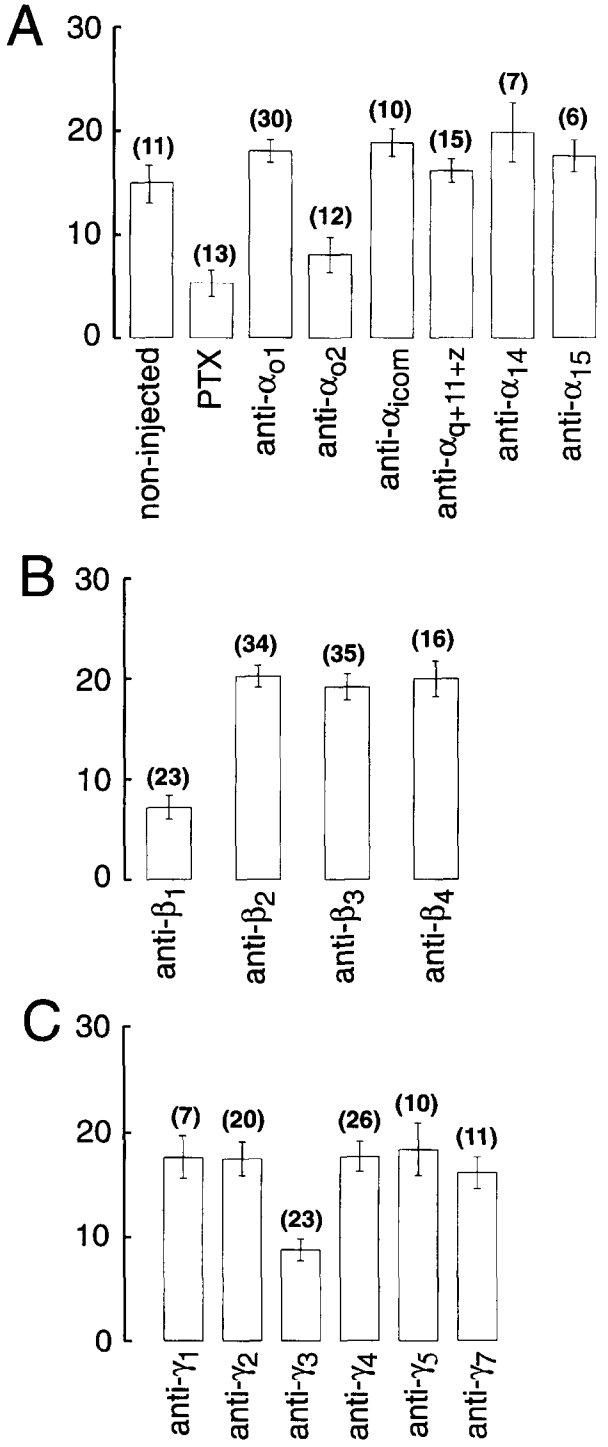
SSDI 0014-5793(96)00047-6

solution of fluorescein isothiocyanate (FITC)-marked oligonucleotides. The fluorescence signal was observed for two days in the nuclei of about 90% of injected cells. Following injection, cells were usually cultured for 44–48 hours before electrophysiological measurements. About 20–60% of the injected cells were suitable for electrophysiological measurements with respect to leak, I_{Ca} amplitude and stability.

2.4. Electrophysiological measurements and data analysis

Glass slides with injected cells were transferred into a perfusion chamber (0.2 ml volume, 4 ml/min perfusion rate) mounted on an inverted microscope. Whole-cell membrane currents were measured at 37°C according to Hamill et al. [15], using a List LM/EPC7 patch-clamp amplifier (List Electronics, Darmstadt, Germany). Patch pipettes were prepared from glass capillaries (Jencons, Leight Buzzard, UK); the average resistance of the pipettes was 2.5–3.5 MΩ. The series resistance was compensated by 40–70%. The mean capacitiy of the RINm5F cells was 13.3 ± 3.9 pF (± S.D., n = 142). Pipettes were filled with Cs^+-containing internal solutions in order to block K^+ conductance (solution I1: 125 mM CsCl, 1 mM $MgCl_2$, 3 mM MgATP, 10 mM EGTA, 10 mM HEPES, pH 7.4 at 37°C, or solution I2 containing: 115 mM CsCl, 1 mM $MgCl_2$, 3 mM MgATP, 20 mM BAPTA, 10 mM HEPES, pH 7.4 at 37°C). Before and after an experiment, cells were superfused with extracellular solution E1 (140 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 1.0 mM $MgCl_2$, 10 mM glucose, 10 mM HEPES, pH 7.4 at 37°C). The whole-cell currents through voltage-gated Ca^{2+} channels (I_{Ca}) were measured using Ba^{2+} as divalent charge carrier in solution E2 (10 mM $BaCl_2$, 1 mM $MgCl_2$, 5.4 mM CsCl, 10 mM glucose, 125 mM *N*-methyl-D-glucamine, 10 mM HEPES, pH 7.4 at 37°C). The maximal amplitude of Ba^{2+} currents was recorded during 20 ms long voltage pulses from the holding potential of −80 mV to 0 mV; the stimulation rate was 0.5 Hz. The Ba^{2+} current amplitude was determined as peak inward current. To take into account run-down of channels, the control current was determined as mean value of Ba^{2+} current amplitudes before somatostatin application and after washout of the hormone. The inhibition of Ba^{2+} current by somatostatin was determined as difference (in percent) between the peak current amplitude of the control current and the peak current amplitude (reached after 6–10 s) during superfusion of the cells with hormone. The significance of the results was determined using Student's t-test assuming a Gaussian distribution of data. Standard errors are given as S.E.M. if not otherwise indicated.



3. Results and discussion

In order to identify the heterotrimeric G proteins coupling somatostatin receptors to voltage-gated Ca^{2+} channels in the rat insulinoma cell line RINm5F, we injected modified antisense oligonucleotides directed against subtypes of G-protein α, β and γ subunits into the nuclei of these cells (Fig. 1). Oligonucleotides carried two phosphorothioate-linked nucleotides at either end with phosphodiester bonds between the other nucleotides. Two days after injection, we measured whole cell Ba^{2+} currents. Somatostatin inhibited the Ba^{2+} current through voltage-gated Ca^{2+}channels (I_{Ca}) by 19% in mean. The same mean inhibition was measured in cells injected with antisense oligonucleotides complementary to the mRNAs encoding for the PTX-insensitive G proteins $G\alpha_{14}$ (anti-α_{14}) and $G\alpha_{15}$ (anti-α_{15}) (Fig. 1A). Injection of a mixture of antisense oligonucleotides directed against the other PTX-insensitive G proteins $G\alpha_q$, $G\alpha_{11}$ and $G\alpha_z$ (anti-α_{q+11+z}) also did not influence inhibition of I_{Ca} by somatostatin. Pretreatment of RINm5F cells with pertussis toxin (100 ng/ml for 24 h) reduced inhibition of I_{Ca} in the same batch of cells from 16% to 5%. Out of the family of PTX

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Fig. 1. Somatostatin-induced Ca^{2+} channel inhibition in RINm5F cells injected with antisense oligonucleotides directed against the mRNAs of G-protein α, β and γ subunits. Ca^{2+} channel inhibition is shown as relative inhibition of the Ba^{2+} current (in%) during superfusion of cells with 1 μM somatostatin. The whole cell Ba^{2+} current was measured 48–72 hours after injection of the cells with oligonucleotides. Numbers in parentheses indicate the number of cells measured for each oligonucleotide. Oligonucleotide sequences were published previously [9–13]. (A) Ba^{2+} current inhibition of RINm5F cells injected with antisense oligonucleotides directed against subtypes of α subunit. PTX indicates cells treated for 24 hours with 100 ng/ml pertussis toxin before measurement of Ba^{2+} currents. (B) Ba^{2+} current inhibition of RINm5F cells injected with antisense oligonucleotides directed against subtypes of β subunit. (C) Ba^{2+} current inhibition of RINm5F cells injected with antisense oligonucleotides directed against subtypes of γ subunit.

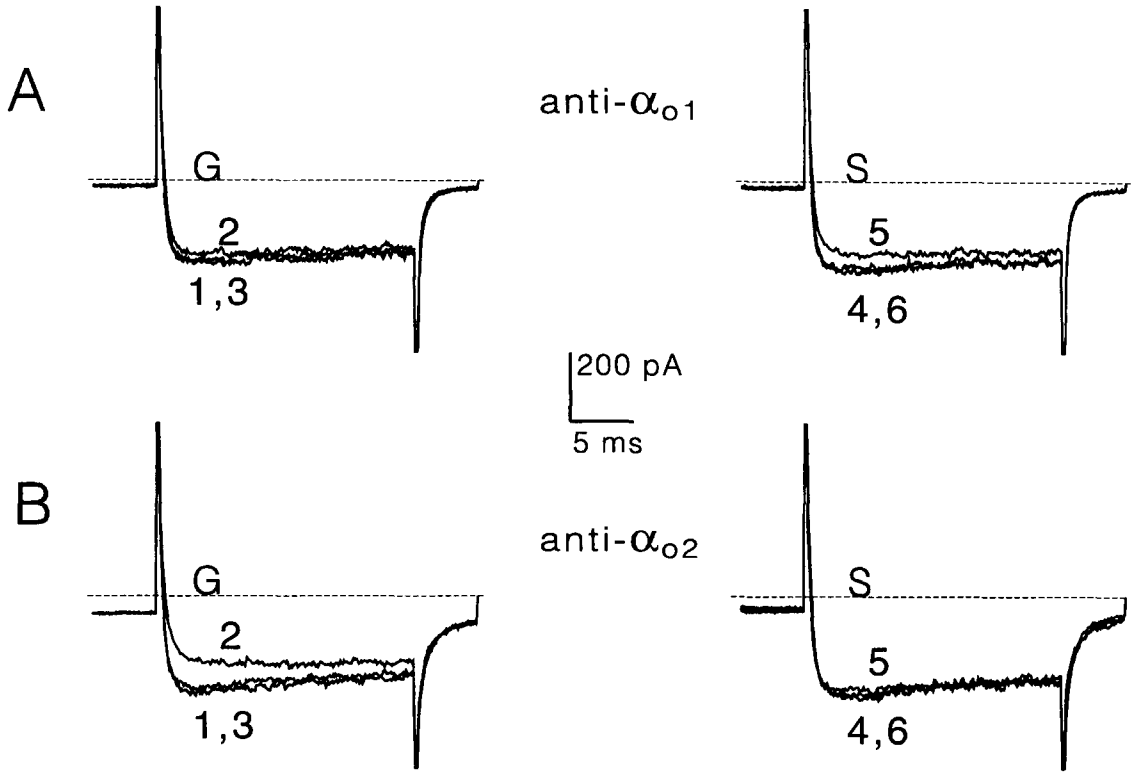

Fig. 2. Recordings of whole cell Ba^{2+} currents in RINm5F cells in the presence of galanin and somatostatin. In each panel, current traces are shown for one cell, each, superfused with either hormone at 44 hours after injection with antisense oligonucleotides anti-α_{o1} or anti-α_{o2}. Each cell was superfused with galanin (G, 500 nM) and somatostatin (S, 1 μM). Traces 1 and 4 are control currents; traces 2 and 5 represent Ba^{2+} currents during superfusion with hormone; traces 3 and 6 represent currents after washing out the agonist. In this experiment, we obtained similar data with 12 anti-α_{o1} injected cells and 5 anti-α_{o2} injected cells; similar data were obtained in additional experiments.

sensitive $G_{i/o}$ proteins the α subunits of G_{i1}, G_{i2}, G_{o1} and G_{o2} are expressed in RINmF5 cells, as shown by Western blots with specific antibodies [8]. Cells injected with antisense oligonucleotides directed against a common sequence of all three subtypes of α subunits of the G_i proteins (anti-α_{icom}) showed unchanged somatostatin-induced inhibition of I_{Ca}. The same holds true for cells injected with antisense oligonucleotides which are designed to anneal selectively to the mRNA coding for the $G\alpha_{o1}$ subunit of G proteins (anti-α_{o1}). In contrast, cells injected with antisense oligonucleotides annealing to the $G\alpha_{o2}$ subunit (anti-α_{o2}) showed reduced inhibition of I_{Ca} by somatostatin compared to control cells (8% vs. 19%, see Fig. 1A). These results indicate that the G protein that mediates the inhibition of voltage-gated Ca^{2+} channels by somatostatin receptors in RINm5F cells includes the $G\alpha_{o2}$ subunit. In cells injected with antisense oligonucleotides annealing to the mRNA encoding the β_1 subunits of G proteins (anti-β_1), the somatostatin-induced inhibition of I_{Ca} was significantly reduced compared to cell injected with antisense oligonucleotides directed against the β_2, β_3 and β_4 subunits of G proteins (anti-β_2, anti-β_3 and anti-β_4, respectively) (Fig. 1B). Among cells injected with antisense oligonucleotides annealing to γ-subunit mRNAs only cells injected with those directed against the γ_3 subunit (anti-γ_3) exibited reduced I_{Ca} inhibition by somatostatin; cells injected with antisense oligonucleotides directed against the γ_1, γ_2, γ_4, γ_5 and γ_7 subunits (anti-γ_1, ant-γ_2, anti-γ_4, anti-γ_5 and anti-γ_7, respectively) displayed a regular degree of somatostatin-induced inhibition of I_{Ca} (Fig. 1C) when compared to non-injected cells (see Fig. 1A).

An important question in using antisense oligonucleotides is the specificity of an effect reached with an antisense oligonucleotide. We previously showed by immunofluorescence studies that in RINm5F cells injected with anti-α_{o1} and anti-α_{o2} oligonucleotides the expression of the $G\alpha_{o1}$ and $G\alpha_{o2}$ proteins, respectively, is selectively inhibited [12]. In addition to the protein expression, we probed the selectivity of the antisense oligonucleotides on the functional level. In cells injected with anti-α_{o2} oligonucleotides, the somatostatin-induced inhibition of I_{Ca} was largely inhibited. In contrast, in the same cells the inhibition of I_{Ca} by galanin was unchanged (Fig. 2B). Recently, we demonstrated that the two G-protein heterotrimers $G\alpha_{o1}\beta_2\gamma_2$ and $G\alpha_{o1}\beta_3\gamma_4$ mediate the galanin-induced inhibition of I_{Ca} in RINmF5 cells [12]. Consequently, in a cell injected with anti-α_{o1} oligonucleotides the galanin-induced inhibition of I_{Ca} was reduced, however, in the same cell the somatostatin-induced inhibition of I_{Ca} was unchanged (Fig. 2A). We also compared the inhibitions of I_{Ca} induced by galanin and somatostatin in RINm5F cells which were injected with antisense oligonucleotides annealing to β- and γ-subunit mRNAs. In anti-β_1-injected cells, the somatostatin-induced inhibition of I_{Ca} was decreased whereas the inhibition induced by galanin was not affected (Fig. 3A). The somatostatin effect remained

unchanged by injection of a mixture of anti-β_2 and anti-β_3 oligonucleotides (anti-β_2 + β_3) (Fig. 3B), but the galanin effect was suppressed in the same cells. Similarly, in cells injected with a mixture of antisense oligonucleotides annealing to γ_2- and γ_4-subunits mRNAs (anti-γ_2 + γ_4) the inhibition of I_{Ca} induced by somatostatin was unchanged (Fig. 3C), but the inhibition

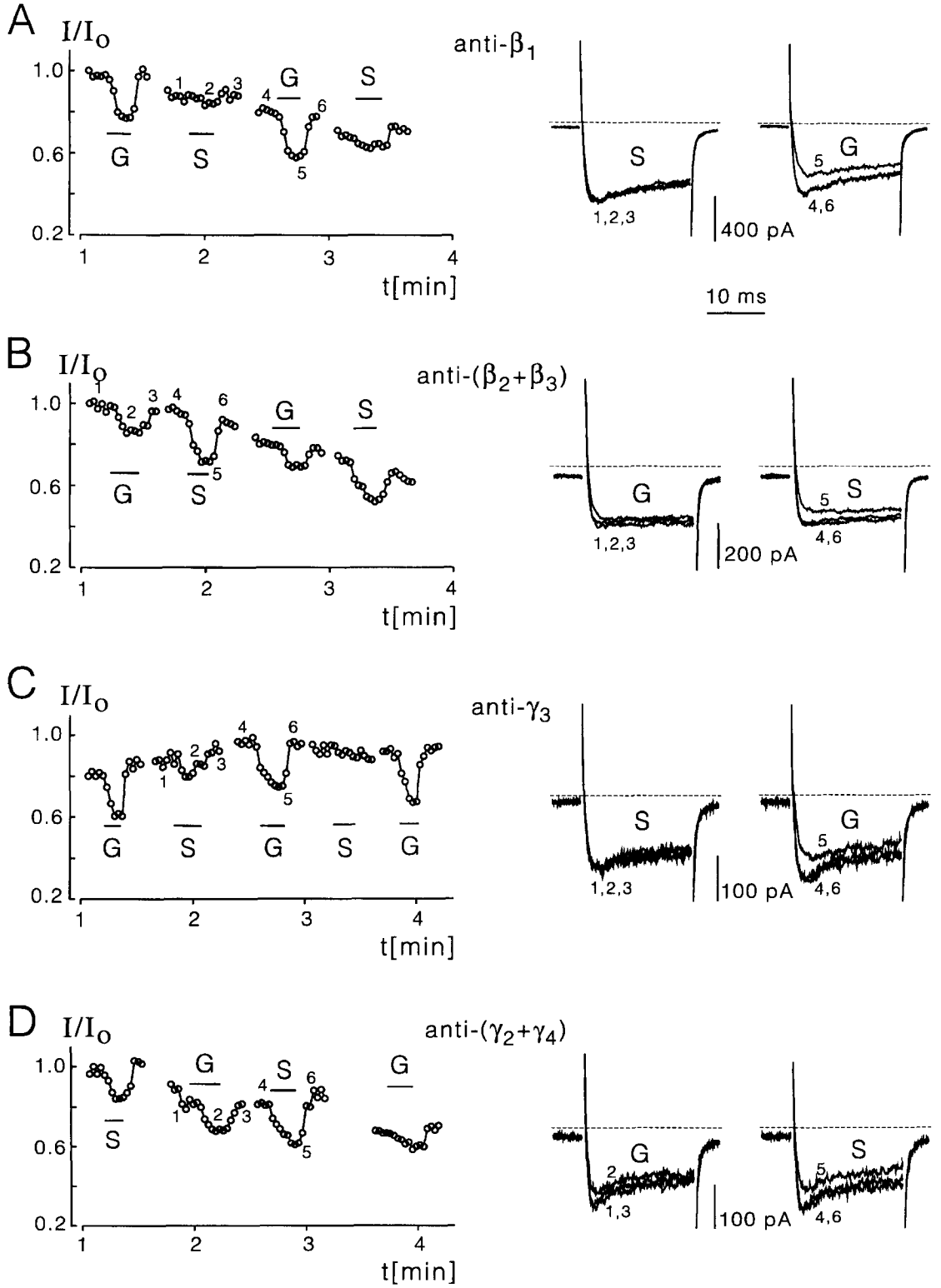

Fig. 3. Somatostatin- and galanin-induced I_{Ba} inhibitions in RINm5F cells injected with antisense oligonucleotides directed against G protein β and γ subunits. I_{Ba} traces (right) and time courses of somatostatin- and galanin-induced inhibitions of I_{Ba} (left) in individual cells injected with anti-β_1 (n = 14 cells) (A), a mixture of anti-β_2 and anti-β_3 (n = 8) (B), anti-γ_3 (n = 17) (C), or a mixture of anti-γ_2 and anti-γ_4 antisense oligonucleotides (n = 7) (D) are shown. Bars denote the time span of galanin (G) or somatostatin (S) application.

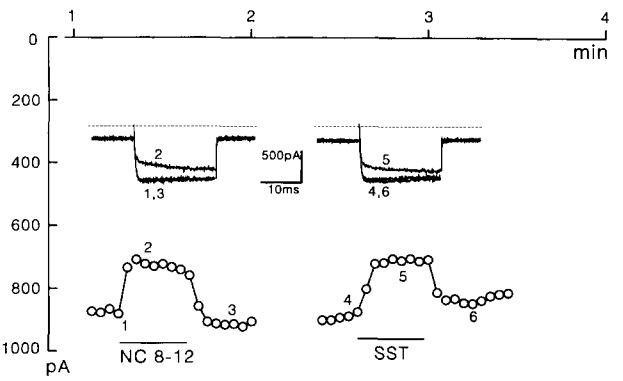

Fig. 4. Current recordings and time courses of whole cell Ba^{2+} currents in RINm5F cells in the presence of the somatostatin analog NC 8–12 and somatostatin. In the upper panels, current traces are shown for one representive cell for each agonist. Traces 1 and 4 are control currents, traces 2 and 5 represent Ba^{2+} currents during superfusion with agonist; traces 3 and 6 represent currents after washing out the agonist. In the lower panels time courses of NC 8–12 and somatostatin-induced I_{Ca} are shown. Numbers refer to the time points from which the traces shown in the upper panels have been taken. We obtained similar data in 4 cells for NC 8–12 and 11 cells for somatostatin.

induced by galanin was reduced. Cells injected with anti-γ_3 oligonucleotides showed reduced responses to somatostatin, but the inhibition of I_{Ca} induced by galanin was not reduced (Fig. 3C). Injection of antisense oligonucleotides annealing to β_4-, γ_1-, γ_5-, and γ_7- subunit mRNAs had no influence on either galanin- or somatostatin-induced inhibition of I_{Ca} (data not shown).

These results demonstrate that the functional knock-out reached by injection of antisense oligonucleotides selectively directed against individual G-protein subunits influenced only the signal transduction pathway in which this particular subunit is involved. It did not touch a pathway mediated by a related hormonal receptor connected to the same effector system, i.e. voltage-gated Ca^{2+} channels.

Taken together, these results indicate that the somatostatin receptors in RINm5F cells use the G protein $G\alpha_{o2}\beta_1\gamma_3$ to inhibit voltage-gated Ca^{2+} channels. Thus, the somatostatin receptor in these cells uses the same G-protein heterotrimer to couple to calcium channels as the one expressed in GH_3 cells [9–11].

To determine which subspecies out of the five known somatostatin receptors is responsible for the inhibition of I_{Ca} in RINm5F cells, we used the somatostatin analog NC 8–12 which selectively activates the somatostatin 2 receptor (SSTR2) [16]. This analog inhibited I_{Ca} in RINm5F cells with the same potency as somatostatin itself (Fig. 4), indicating that SSTR2 may be the receptor subtype mediating somatostatin-induced inhibition of voltage-gated Ca^{2+} channels in RINm5F cells. These findings are in accordance with data of Fuji et al. [17]. They stably transfected the human SSTR2 gene into a derivative RINm5F line lacking endogenous somatostatin receptor expression and thereby restored coupling to voltage-gated Ca^{2+} channels.

Acknowledgements: We thank Rita Hauboldt for excellent technical assistance, Katrin Büttner for synthesis of oligonucleotides, Dr. David H. Coy (New Orleans, USA) for providing the somatostatin analog NC 8–12. This work was supported by grants of the Deutsche Forschungsgemeinschaft and Fonds der Chemischen Industrie.

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