Different G proteins mediate somatostatin-induced inward rectifier K^+ currents in murine brain and endocrine cells

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1. Types of G proteins (G protein α-subunit subtypes) which mediate the activation of inward rectifier K^+ currents by somatostatin (somatotrophin release-inhibiting factor, SRIF) were determined in cultured locus coeruleus neurones from newborn rats and in AtT-20 cells (a mouse pituitary cell line).

2. The whole-cell patch clamp technique was used together with injection of antibodies against pertussis toxin (PTX)-sensitive G protein α-subunits or with injection of antisense (or sense) oligonucleotides against these G proteins.

3. In locus coeruleus neurones, the SRIF-induced activation of inward rectifier K^+ currents was inhibited by anti-$G\alpha_{i1}$/$G\alpha_{i2}$ antibody injection, but not by anti-$G\alpha_{i3}$ or by anti-$G\alpha_o$/$G\alpha_{i3}$ antibody injection, suggesting that the SRIF response is mediated through $G\alpha_{i1}$ and/or $G\alpha_{i2}$.

4. The SRIF-induced activation of the inward rectifier was suppressed in locus coeruleus neurones after injection of antisense oligonucleotides against $G\alpha_{i2}$, but not by injection of sense oligonucleotides against $G\alpha_{i2}$. Injection of antisense (or sense) oligonucleotides against $G\alpha_{i1}$, $G\alpha_{i3}$ and $G\alpha_o$(common) had no effect. These results suggest that $G\alpha_{i2}$ is involved in this SRIF response.

5. In AtT-20 cells, the SRIF-induced activation of inward rectifier K^+ currents was suppressed by injection of anti-$G\alpha_{i3}$ antibody, but not by injection of anti-$G\alpha_{i1}$/$G\alpha_{i2}$ antibody.

6. The above results indicate that G_i mediates the SRIF effects on inward rectifier K^+ currents. However, different subtypes of G_i are involved in the brain neurones and in the endocrine cells: G_{i2} in locus coeruleus neurones and G_{i3} in AtT-20 cells.

Somatostatin (somatotrophin release-inhibiting factor, SRIF), a fourteen amino acid peptide, was discovered in the hypothalamus (Brazeau *et al.* 1973). This peptide was shown to inhibit the secretion of growth hormone in the hypophysis (Brazeau *et al.* 1973). Later, SRIF was shown to be widely distributed in the brain, and it is now known to be an important neurotransmitter in the brain.

The locus coeruleus, a pair of small nuclei located in the brainstem, consists of tightly packed noradrenergic neurones which project to virtually the entire brain and spinal cord. Hence, this nucleus, as the main supplier of noradrenaline in the brain, plays a vital role in brain function. SRIF inhibits locus coeruleus neurones by activating an inward rectifier K^+ current, and this SRIF effect is mediated through a pertussis toxin (PTX)-sensitive G protein (Inoue, Nakajima & Nakajima, 1988). However, the type (or types) of PTX-sensitive G protein (G_{i1}, G_{i2}, G_{i3} or G_o) involved in this SRIF effect has yet to be determined.

In AtT-20 cells (a mouse pituitary tumour cell line) and in human pituitary tumour cells, SRIF activates an inward rectifier K^+ current, and this SRIF effect is also mediated by a PTX-sensitive G protein (Yamashita, Shibuya & Ogata, 1988; Pennefather, Heisler & MacDonald, 1988; Kozasa, Kaziro, Ohtsuka, Grigg, Nakajima & Nakajima, 1996). In AtT-20 cells as well, it has not yet been determined which one of several PTX-sensitive G proteins is involved in the SRIF effect. The objective of this paper is to identify the G protein that mediates the SRIF effect on inward rectifier K^+ currents in locus coeruleus neurones and in AtT-20 cells. To accomplish this, we intracellularly injected antibodies or

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antisense oligonucleotides against PTX-sensitive G protein α-subunits using an Eppendorf microinjector, and then conducted electrophysiological experiments.

METHODS

Cell cultures of locus coeruleus neurones

Locus coeruleus neurones were cultured from 2- to 3-day-old postnatal Long–Evans rats (Charles River Laboratories, Inc.), using the method previously reported (Masuko, Nakajima, Nakajima & Yamaguchi, 1986), except that the neurones were dissociated with papain instead of trypsin and the culture medium contained rat serum instead of fetal bovine serum. Under ether anaesthesia, brainstems, from the pons to the upper region of the cervical spinal cord, were totally removed. The animals were killed by this procedure, which corresponds to decapitation. The brainstems were embedded in agar, and brain slices (300–400 μm) were obtained using a vibratome (Lancer 1000). The locus coeruleus was visually identified under a dissecting microscope and isolated from the slices. After the isolated pieces of locus coeruleus were treated with papain (12 units ml^{-1}), they were dissociated, and plated on a glial feeder layer. The culture medium was Eagle's minimum essential medium containing Earle's salts (no. 330-1430, Gibco) modified by the addition of L-glutamine (0·292 mg ml^{-1}), $NaHCO_3$ (3·7 mg ml^{-1}) and D-glucose (5 mg ml^{-1}), and was supplemented with heat-inactivated rat serum (2%) (prepared in our laboratory), L-ascorbic acid (10 μg ml^{-1}), penicillin (50 units ml^{-1}) and streptomycin (50 μg ml^{-1}). The cultures were incubated at 37 °C in air containing 10% CO_2, usually for 14–21 days. Experiments were performed on large neurones (26–38 μm in diameter), which were likely to be noradrenergic neurones (Masuko *et al.* 1986).

Cell cultures of AtT-20 cells

AtT-20 cells (provided by Dr Deborah L. Lewis, Medical College Georgia, Augusta, GA, USA) were subcultured every week in tissue culture dishes in Dulbecco's modified Eagle's Medium (no. 320-1960AJ, Gibco) with 10% fetal bovine serum, 2 mM glutamine, 50 units ml^{-1} penicillin and 50 μg ml^{-1} streptomycin (Lewis, Weight & Luini, 1986). The cultures were kept at 37 °C in humidified air containing 10% CO_2, and the medium was changed every fourth day. For physiological experiments, cells were plated in 35 mm tissue culture dishes, and the experiments were done on the cells after 3 days of subculture.

Electrophysiology

The whole-cell patch clamp was performed using thoroughly washed and Sylgard-coated patch pipettes made from hard glass tubing (7052 glass, Garner Glass Co., Claremont, CA, USA). During the experiments, cells were continuously superfused with an oxygenated external solution. The standard external solution (5 mM K^+ Krebs solution) contained (mM): 146 NaCl, 5 KCl, 2·4 $CaCl_2$, 1·3 $MgCl_2$, 5 Hepes–NaOH buffer, 11 D-glucose and 0·0005 tetrodotoxin (pH 7·4). The standard internal solution contained (mM): 144 potassium aspartate, 10 NaCl, 3 $MgCl_2$, 0·25 $CaCl_2$, 0·5 EGTA–KOH, 2 Na_2ATP, 0·1 Na_3GTP, 5 Hepes–KOH and ~5 KOH (pH 7·2). SRIF was dissolved in the external solution and applied by pressure ejection (5–7 kPa) through a thoroughly washed micropipette (drug pipette) made from soft glass (R6 glass, Garner Glass Co.). The tips of the drug pipettes were 4–6 μm in diameter, and the distance between the tip and the soma was ~40 μm. When not in use, the drug pipette was kept in the air to prevent contamination of the external solution. The records were stored on computer and videotape using a pulse code modulator (VR-10A, Instrutech Corp., Great Neck, NY, USA), and were later analysed with pCLAMP programs (Axon Instruments). In almost all experiments the series resistance was partially compensated for electronically, and the error caused by the remaining resistance was corrected for at the data analysis stage. The membrane potential was corrected for the liquid junction potential between the standard internal and external solutions (12 mV, external solution positive). All statistical values were expressed as means ± S.D. The conductances were not normalized to cell size. Experiments were performed at ~22 °C.

Chemicals

Somatostatin (SRIF) was purchased from Peninsula (Belmont, CA, USA).

Antibodies against G proteins

Three kinds of polyclonal antibodies against the carboxyl terminus peptides of $G\alpha_i$ and $G\alpha_o$ were used. They were: (1) anti-$G\alpha_{i1}/G\alpha_{i2}$ antibody (2·8 mg ml^{-1}, affinity purified) (no. 371723, Calbiochem), which was developed against a peptide representing amino acids 345–354 of the common carboxyl termini of $G\alpha_{i1}$ and $G\alpha_{i2}$, and which is specific to both $G\alpha_{i1}$ and $G\alpha_{i2}$ (Goldsmith *et al.* 1987; Mumby & Gilman, 1991); (2) anti-$G\alpha_{i3}$ antibody (4·2 mg ml^{-1}, affinity purified) (no. 371729, Calbiochem), which was developed against a peptide representing amino acids 345–354 of the carboxyl terminus of $G\alpha_{i3}$, and which is monospecific to $G\alpha_{i3}$; and (3) anti-$G\alpha_o/G\alpha_{i3}$ antibody (undiluted antiserum) (no. 371726, Calbiochem), which was developed against a peptide representing amino acids 345–354 of the carboxyl termini of $G\alpha_o$ and $G\alpha_{i3}$, and which is specific to both $G\alpha_o$ and $G\alpha_{i3}$ (Jones & Reed, 1987; Mumby & Gilman, 1991). Since the pre-immune IgG for the above-mentioned antibodies (obtained from Calbiochem) was not available, we used pre-immune IgG for $G\alpha_{q/11}$ antibody (2–6 mg ml^{-1}) obtained from Dr P. C. Sternweis (University of Texas, Southwestern Medical Centre at Dallas) as the 'control IgG' for the antibodies.

Antisense and sense oligonucleotides to $G\alpha_i$ and $G\alpha_o$ subunits

Two types of antisense and sense oligonucleotides were used to $G\alpha_{i1}$.

Antisense-$G\alpha_{i1}$(1): 5′-CATGGTGGCCGACGTCGCCCGC CCTCGGCGCCGGGGCCG-3′.

Sense-$G\alpha_{i1}$(1): 5′-CGGCCCCGGCGCCGAGGGCGGGC GACGTCGGCCACCATG-3′.

(Based on 5′-non-coding sequence upstream of the initiation codon of rat $G\alpha_{i1}$ cDNA; H. Itoh, T. Kozasa & Y. Kaziro, unpublished data.)

Antisense-$G\alpha_{i1}$(2): 5′-CATGGTGGCCGAGCGTCGCCGC GCCCTCGGCGCCGGGGC-3′.

Sense-$G\alpha_{i1}$(2): 5′-GCCCCGGCGCCGAGGGCGCGGCG ACGCTCGGCCACCATG-3′.

(From the leader sequences just before the initiation codon, based on the rat $G\alpha_{i1}$ cDNA sequence; Jones & Reed, 1987.)

Antisense-$G\alpha_{i2}$: 5′-CATCCTGCCGTCCGCCGGCCCGGC CTGGCCCCCACCACG-3′.

Sense-$G\alpha_{i2}$: 5′-CGTGGTGGGGGCCAGGCCGGGCCGG CGGACGGCAGGATG-3′.

(From the leader sequences just before the initiation codon, based on the rat $G\alpha_{i2}$ cDNA sequence; Itoh, Toyama, Kozasa, Tsukamoto, Matsuoka & Kaziro, 1988.)

Antisense-$G\alpha_{i3}$: 5′-CATGACGGCGGCCGGAGAGGGGA CCGGGCCCTGGCTCAC-3′.

Sense-$G\alpha_{i3}$: 5′-GTGAGCCAGGGCCCGGTCCCCTCTCC GGCCGCCGTCATG-3′

(From the leader sequences just before the initiation codon, based on the rat $G\alpha_{i3}$ cDNA sequence; Itoh *et al.* 1988.)

Antisense-$G\alpha_o$(common): 5′-CATGGTGGCCCCT TCCCTG CCACAGCCCGCACGACTCGG-3′.

Sense-$G\alpha_o$(common): 5′-CCGAGTCGTGCGGGCTGTGGC AGGGAAGGGGCCACCATG-3′.

(From the leader sequences just before the initiation codon, based on the rat $G\alpha_o$ cDNA sequence; Jones & Reed, 1987; these nucleotides are similar to the one used by Kleuss, Hescheler, Ewel, Rosenthal, Schultz & Wittig, 1991.)

The sequences of the oligonucleotides were checked and had no dimer formation, no hairpin structure and no inverted repeat sequences. No significant similarities to other sequences were detected by searching available databases (Genbank, EMBL, PDB and DDBJ). According to the database search, the probability of a match between each of the oligonucleotides and its corresponding $G\alpha$ is 10^6–10^7 times higher than the probability of a match between the oligonucleotide and other sequences. Therefore, each oligonucleotide sequence is specific for its $G\alpha$ subunit and hybridization with other sequences is unlikely.

All oligonucleotides were HPLC purified but were not phosphorothioate modified. All oligonucleotides were used at 10 μM in 140 mM KCl.

Intracellular microinjection of antibodies and oligonucleotides

We injected the antibodies and oligonucleotides into the cytoplasm through microcapillaries (Femtotips, Eppendorf) by using an Eppendorf microinjector (5242) and micromanipulator (5171) system, as previously described (Takano, Stanfield, Nakajima & Nakajima, 1995; Takano, Yasufuku-Takano, Kozasa, Singer, Nakajima & Nakajima, 1996). In the antibody experiments, we used 160 hPa pressure for 0·3 s for locus coeruleus neurones, and 90–110 hPa for 0·1 s for AtT-20 cells.

In oligonucleotide injection (only locus coeruleus neurones were used) the injection pressure was 140 hPa for 0·3 s. This pressure is larger than that used by Kleuss *et al.* (1991) (20–30 hPa for 0·1 s) in their oligonucleotide injection experiments in GH_3 (clonal pituitary) cells. However, locus coeruleus neurones are much larger than GH_3 cells; in the present study the diameter (geometrical mean of larger and smaller diameters of the soma) of locus coeruleus neurones was 26–38 μm, while GH_3 cell diameter is only 14 μm (Dubinsky & Oxford, 1984), and therefore the volume of locus coeruleus neurones could be 6–20 times greater than that of GH_3 cells. Thus, the

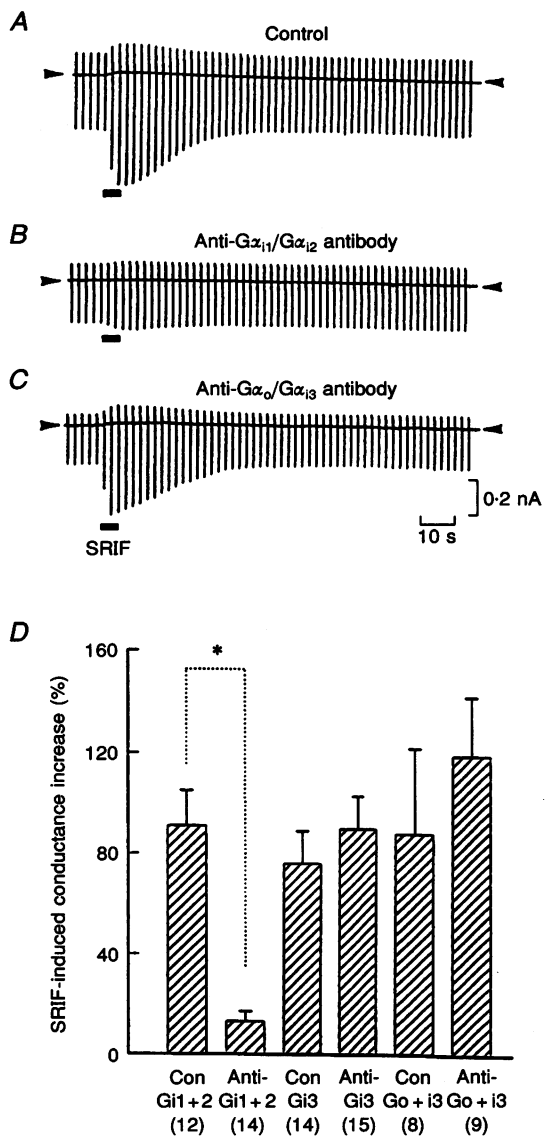

Figure 1. Effects of anti-$G\alpha_{i1}$/$G\alpha_{i2}$ antibody, anti-$G\alpha_{i3}$ antibody and anti-$G\alpha_o$/$G\alpha_{i3}$ antibody on SRIF (0·3 μM)-induced responses in locus coeruleus neurones

Each antibody was pressure injected with an Eppendorf microinjector. Whole-cell voltage clamp recordings were obtained from the injected neurones 50–140 min after injection. The membrane conductance was monitored by applying recurrent command pulse sequences, each consisting of a depolarizing pulse (20 mV, 100 ms) followed by a 100 ms interval and a hyperpolarizing pulse (−50 mV, 100 ms). The holding potential was −77 mV. Arrowheads indicate the zero current level. *A*, neurone injected with control IgG (see Methods). *B*, neurone injected with anti-$G\alpha_{i1}$/$G\alpha_{i2}$ antibody. *C*, neurone injected with anti-$G\alpha_o$/$G\alpha_{i3}$ antibody. *D*, summary of antibody experiments. The ordinate is the peak conductance increase (%) induced by SRIF with reference to the membrane conductance before SRIF application. The SRIF-induced conductance increase was significantly suppressed (* significant difference, $P < 0{\cdot}0001$) in neurones injected with anti-$G\alpha_{i1}$/$G\alpha_{i2}$ antibody (anti-Gi1 + 2) compared with the control neurones (Con Gi1 + 2). On the other hand, SRIF-induced responses in neurones injected with anti-$G\alpha_{i3}$ antibody (anti-Gi3) and the responses in neurones injected with anti-$G\alpha_o$/$G\alpha_{i3}$ antibody (anti-Go + i3) were not significantly different ($P = 0{\cdot}05$) from the responses of the control neurones (Con Gi3 and Con Go + i3). All control neurones were injected with control IgG. One-way ANOVA was used. Error bars indicate S.D. The number of experiments is listed beneath each column. We also examined the SRIF response in non-injected neurones and found that there was no significant difference in SRIF responses between these non-injected neurones (120 ± 28%, $n = 6$) and control IgG-injected neurones (91 ± 14%, $n = 12$).

relative amount of injected oligonucleotides in the present study would not be very different from that in the experiments of Kleuss *et al.* (1991).

The injected cells were identified by marking the bottom of the culture dish with a hypodermic needle, and physiological recordings were obtained from the injected cells (50–140 min after antibody injection, and 37–43 h after antisense oligonucleotide injection).

In experiments using locus coeruleus neurones, the SRIF response varied among different batches of culture. Therefore, for each batch approximately the same number of test neurones and control neurones were sampled.

RESULTS

Effects of antibodies on the SRIF response in locus coeruleus neurones

Our previous studies showed that SRIF inhibits locus coeruleus neurones by inducing a G protein-mediated inward rectifier K^+ current (K_{IR}(G) current), and that the G protein involved is PTX sensitive (Inoue *et al.* 1988). To determine the type of PTX-sensitive G protein, anti-Gα_{i1}/Gα_{i2} antibody, anti-Gα_{i3} antibody and anti-Gα_o/Gα_{i3} antibody were injected into neurones and 50–140 min later the response to SRIF (0·3 μM) was examined. All these antibodies are against the carboxyl terminus peptides of G protein α-subunits; the carboxyl terminus is the domain for receptor–G protein interaction (Kaziro, Itoh, Kozasa, Nakafuku & Satoh, 1991). As controls, we injected 'control IgG' (see Methods) into neurones. Figure 1*A* shows the effect of SRIF on such a control neurone. Recurrent depolarizing (20 mV, 100 ms) and hyperpolarizing (−50 mV, 100 ms) command voltage pulses were applied from a holding potential of −77 mV to monitor the membrane conductance. SRIF induced a conductance increase accompanied by an outward shift of the holding current (Fig. 1*A*). As was reported previously, this response represents enhancement of the K_{IR}(G) current (Inoue *et al.* 1988; Grigg, Kozasa, Nakajima & Nakajima, 1996). In contrast, SRIF induced only a very slight conductance increase in neurones injected with anti-Gα_{i1}/Gα_{i2} antibody, as shown in Fig. 1*B*. SRIF did, however, induce a conductance increase in neurones injected with anti-Gα_o/Gα_{i3} antibody (Fig. 1*C*) and in neurones injected with anti-Gα_{i3} antibody.

Figure 1*D* summarizes the effects of antibody injection on the SRIF-induced conductance increase with reference to the conductance before SRIF application. Only in cells injected with anti-Gα_{i1}/Gα_{i2} antibody was the response greatly reduced (13 ± 4%; mean ± S.D., $n = 14$) compared with that in control IgG-injected neurones (91 ± 14%, $n = 12$). The SRIF response in cells injected with anti-Gα_{i3} antibody or with anti-Gα_o/Gα_{i3} antibody was no different from the SRIF response in control cells. These results

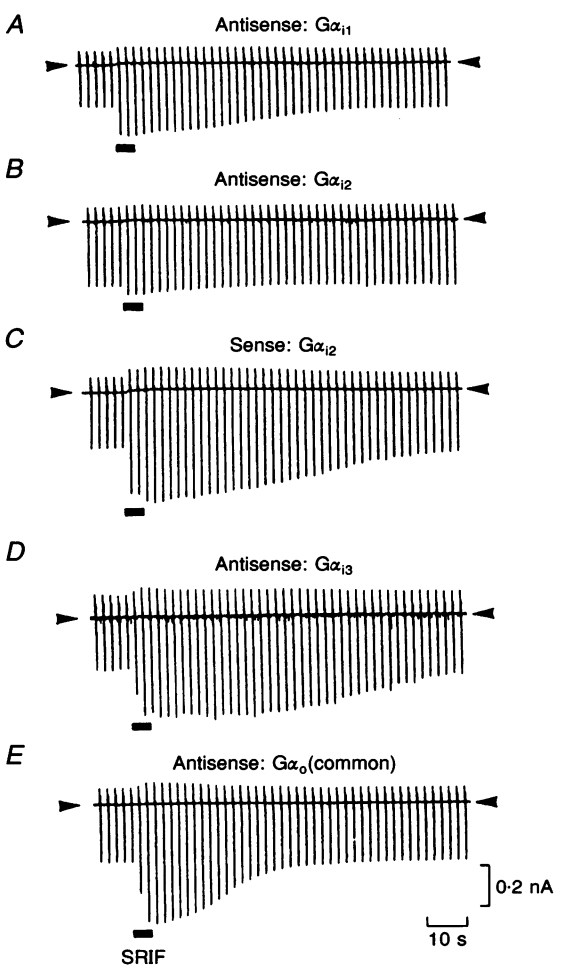

Figure 2. Effects of antisense oligonucleotides against G proteins Gα_{i1}, Gα_{i2}, Gα_{i3} and Gα_o on SRIF (0·3 μM)-induced responses in locus coeruleus neurones

The oligonucleotide (10 μM in 140 mM KCl) was pressure injected with an Eppendorf microinjector. About 40 h after injection, whole-cell voltage clamp recordings were obtained from the injected neurones. The membrane conductance was monitored by applying recurrent command pulse sequences (described in Fig. 1 legend). The holding potential was −77 mV. Arrowheads indicate the zero current level. *A*, neurone injected with antisense oligonucleotides against Gα_{i1}(1) (see Methods). *B*, neurone injected with antisense oligonucleotides against Gα_{i2}. *C*, neurone injected with sense oligonucleotides against Gα_{i2}. *D*, neurone injected with antisense oligonucleotides against Gα_{i3}. *E*, neurone injected with antisense oligonucleotides against Gα_o(common).

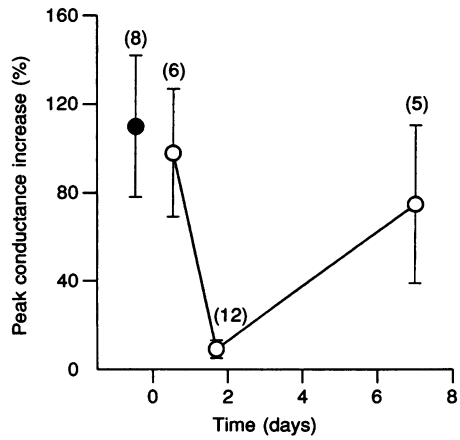

Figure 3. Time course of the effect of injected antisense oligonucleotides against $G\alpha_{i2}$ on the SRIF (0·3 μM)-induced increase in membrane conductance

The ordinate is the peak conductance increase (%) induced by SRIF with reference to the membrane conductance before SRIF application, and the abscissa is time after oligonucleotide injection. The number of experiments is listed above each point. ●, control data from uninjected cells. Error bars indicate S.D.

suggest that the SRIF response in locus coeruleus neurones is mediated by G_{i1} or G_{i2} or both, but not by G_{i3} or G_o.

Effects of antisense (or sense) oligonucleotides on the SRIF response in locus coeruleus neurones

Since $G\alpha_{i1}$ and $G\alpha_{i2}$ cannot be differentiated using antibodies, we used antisense oligonucleotides against $G\alpha_{i1}$ and against $G\alpha_{i2}$. In addition, we used antisense oligonucleotides against $G\alpha_{i3}$ and $G\alpha_o$(common) to confirm the results obtained with antibodies. As controls, we used sense oligonucleotides. Oligonucleotides were pressure injected with a microinjector, and 37–43 h after injection, electrophysiological experiments were performed. In neurones injected with antisense oligonucleotides against $G\alpha_{i1}$(1) (see Methods), SRIF (0·3 μM) induced a conductance increase accompanied by an outward current (Fig. 2*A*). In contrast, in neurones injected with antisense oligonucleotides against $G\alpha_{i2}$, SRIF hardly induced a conductance increase (Fig. 2*B*). In neurones injected with sense oligonucleotides against $G\alpha_{i2}$ (as a control to antisense oligonucleotide injection) the conductance increase by SRIF was not attenuated (Fig. 2*C*). SRIF also induced a conductance increase in neurones injected with antisense oligonucleotides against $G\alpha_{i3}$ (Fig. 2*D*) or against $G\alpha_o$(common) (Fig. 2*E*). Figure 3 shows the time course of the SRIF effect after intracellular injection of antisense oligonucleotides against $G\alpha_{i2}$. The conductance increase by SRIF was largely suppressed ~40 h after oligonucleotide injection, and it partially recovered 7 days after injection.

Figure 4 shows the SRIF-induced conductance increase with reference to the conductance before SRIF application. The whole-cell patch experiments were performed 37–43 h after oligonucleotide injection. The SRIF-induced response in neurones injected with antisense oligonucleotides against $G\alpha_{i2}$ ($9 \pm 4\%$, $n = 12$) was much smaller than that in neurones injected with sense oligonucleotides against $G\alpha_{i2}$ ($90 \pm 14\%$, $n = 11$). On the other hand, no significant difference was observed in the SRIF response between neurones injected with antisense and sense oligonucleotides against $G\alpha_{i1}$, $G\alpha_{i3}$ or $G\alpha_{io}$(common). These results suggest that $G\alpha_{i2}$ mediates the SRIF effects on K_{IR}(G). As described in Methods, we used two different antisense and sense oligonucleotides against $G\alpha_{i1}$ ($G\alpha_{i1}$(1) and $G\alpha_{i1}$(2)). In Fig. 4, the results using antisense and sense oligonucleotides against $G\alpha_{i1}$(1) are shown. We also obtained similar results using antisense and sense oligonucleotides against $G\alpha_{i1}$(2): the

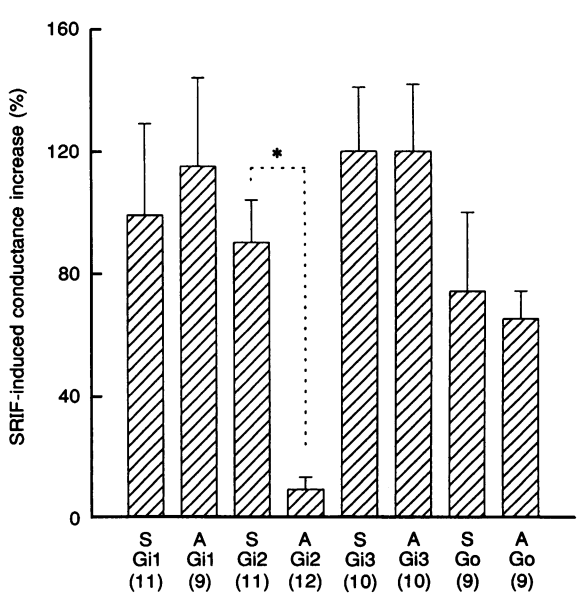

Figure 4. Summary of oligonucleotide experiments

Data were obtained 37–43 h after oligonucleotide injection. The percentage increase in membrane conductance measured at the peak of the SRIF (0·3 μM) effect compared with the conductance before SRIF application is shown for antisense (A) or sense (S) oligonucleotides against $G\alpha_{i1}$ (Gi1), $G\alpha_{i2}$ (Gi2), $G\alpha_{i3}$ (Gi3) and $G\alpha_o$(common) (Go). The number of experiments is listed beneath each column. * Significant difference ($P < 0{\cdot}01$). One-way ANOVA was used. Error bars indicate S.D. Antisense-$G\alpha_{i1}$(1) and sense-$G\alpha_{i1}$(1) oligonucleotides (see Methods) were used in the experiments shown in this figure.

SRIF-induced responses in neurones injected with antisense and sense oligonucleotides against $G\alpha_{i1}(2)$ were 82 ± 20% ($n = 5$) and 104 ± 42% ($n = 5$), respectively. The absence of an effect of antisense oligonucleotides against $G\alpha_{i3}$ or $G\alpha_o$(common) on the SRIF-induced inward rectifier K^+ current agrees with the results obtained using antibodies against $G\alpha_{i3}$ and $G\alpha_o/G\alpha_{i3}$.

Effects of antibodies on the SRIF response in AtT-20 cells

AtT-20 cells, a mouse pituitary tumour cell line, responded to SRIF by inducing the inward rectifier K^+ current, and this response is also mediated through a PTX-sensitive G protein (Pennefather *et al.* 1988; Kozasa *et al.* 1994). To identify which type of PTX-sensitive G protein is involved in this SRIF response, a whole-cell patch clamp study was conducted after antibodies were injected into AtT-20 cells. Figure 5*A* shows a representative SRIF response of an AtT-20 cell which was injected with antibody against $G\alpha_{i1}/G\alpha_{i2}$. The cell responded to SRIF with a conductance increase. In contrast, as shown in Fig. 5*B*, SRIF induced almost no response in cells injected with the antibody against $G\alpha_{i3}$. Figure 5*C* shows the percentage increase in membrane conductance measured at the peak of the SRIF effect with reference to the conductance before SRIF application in cells injected with control IgG, anti-$G\alpha_{i1}/G\alpha_{i2}$ antibody, and anti-$G\alpha_{i3}$ antibody. The mean conductance increase induced by SRIF in cells injected with anti-$G\alpha_{i1}/G\alpha_{i2}$ antibody (209 ± 38%, $n = 9$) was not attenuated but rather was enhanced ($P < 0.01$) compared with the control response (119 ± 20%, $n = 9$). The SRIF response was greatly reduced ($P < 0.01$) in cells injected with anti-$G\alpha_{i3}$ antibody (24 ± 7%, $n = 9$) compared with the control response. These results suggest that the SRIF-induced activation of the K_{IR}(G) current in AtT-20 cells is mediated by G_{i3}. The result that the injection of anti-$G\alpha_{i1}/G\alpha_{i2}$ significantly increased the effect of SRIF is puzzling. One way of interpreting these data is that the inward rectifier K^+ current could be tonically inhibited by an unknown mechanism through G_{i1} and/or G_{i2} in AtT-20 cells. In a separate series of experiments, we co-injected anti-$G\alpha_{i1}/G\alpha_{i2}$ antibody together with anti-$G\alpha_{i3}$ antibody (1 : 1 ratio), and found that the SRIF response was suppressed in cells injected with this mixture of antibodies (34 ± 7%, $n = 9$) compared with that in non-injected cells (154 ± 70%, $n = 9$), suggesting that the effect of G_{i3} dominates that of G_{i1}/G_{i2}.

DISCUSSION

SRIF response in locus coeruleus neurones

It is established that G_o (not G_i) is involved in the transmitter-induced inhibition of voltage-gated Ca^{2+} currents (Kleuss *et al.* 1991; Campbell, Berrow & Dolphin, 1993; Caulfield *et al.* 1994). In contrast, the situation of the transmitter-induced activation of K_{IR}(G) current is less clear. Earlier experiments showed that purified $G\alpha_i$ activated K_{IR}(G) current in atrial cells (Yatani, Codina,

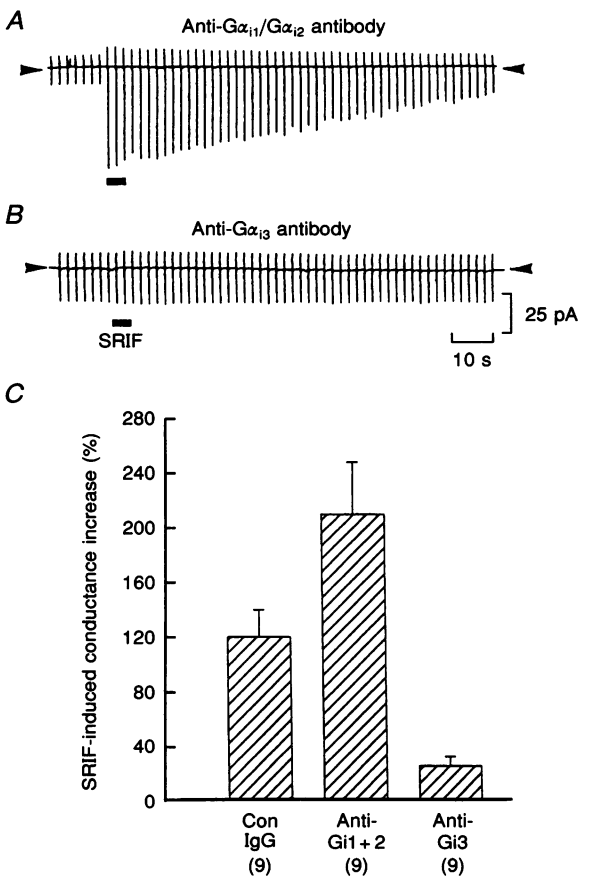

Figure 5. Experiments on AtT-20 cells: effects of anti-$G\alpha_{i1}/G\alpha_{i2}$ antibody and anti-$G\alpha_{i3}$ antibody on SRIF (0·3 μM)-induced responses

One to two hours after antibody injection, whole-cell voltage clamp recordings were obtained from the injected cells. The membrane conductance was monitored by applying recurrent command pulse sequences as described in Fig. 1 legend. The holding potential was −77 mV. Arrowheads indicate the zero current level. *A*, AtT-20 cell injected with anti-$G\alpha_{i1}/G\alpha_{i2}$ antibody responded to SRIF (0·3 μM) with an increased conductance. *B*, AtT-20 cell injected with anti-$G\alpha_{i3}$ antibody hardly responded to SRIF. *C*, summary of antibody experiments. The percentage increase in membrane conductance measured at the peak of the SRIF effect compared with the conductance before SRIF application was obtained for cells injected with control IgG (Con IgG), anti-$G\alpha_{i1}/G\alpha_{i2}$ antibody (Anti-Gi1 + 2) and anti-$G\alpha_{i3}$ antibody (Anti-Gi3). The number of experiments is listed beneath each column. One-way ANOVA was used. Error bars indicate S.D.

Brown & Birnbaumer, 1987). Recent experiments using recombinant G proteins, however, have indicated that the $\beta\gamma$-subunits rather than the α-subunit activate $K_{IR}(G)$ current (Reuveny *et al.* 1994; Wickman *et al.* 1994), and the question of the identity of the α-subunit in the transmitter-induced activation of $K_{IR}(G)$ current remained unanswered.

The identity of G proteins in transmitter-induced modulation of ion channels has been determined either by using antibodies, which are specific to certain G proteins, or by using antisense oligonucleotides. In the present experiments on locus coeruleus neurones, we used both the antibody technique and the antisense oligonucleotide technique. The two sets of data using these different approaches were consistent and complement each other. We used eight different oligonucleotides (either antisense or sense oligonucleotides against $G\alpha_{i1}$, $G\alpha_{i2}$, $G\alpha_{i3}$ and $G\alpha_o$), and found that none of the oligonucleotides, except one, produced noticeable effects on the SRIF-induced response, indicating that the injection of the oligonucleotides *per se* did not produce unspecific effects. The only exception was the injection of an antisense oligonucleotide against $G\alpha_{i2}$, which drastically suppressed the SRIF effect. This result was consistent with the data of antibody experiments, in which out of six different antibodies, only one (the anti-$G\alpha_{i1}/G\alpha_{i2}$) drastically blocked the SRIF effect on $K_{IR}(G)$ current. Thus, the present two sets of results, taken together, lead to the conclusion that G_{i2} (not G_o) is involved in the signal transduction of the SRIF effect on $K_{IR}(G)$ current.

We used non-modified oligonucleotides (not phosphorothioate modified) in the present experiments. According to Fisher, Terhorst, Cao & Wagner (1993), unmodified oligonucleotides are degraded quite rapidly with a half-time of 15–20 min. Nevertheless, Kleuss *et al.* (1991) did find the inhibition of gene expression of $G\alpha_o$ 2 days after the injection of non-modified antisense oligonucleotides with an Eppendorf microinjector.

Molecular biological studies have revealed five types of SRIF receptors (SSTR1, SSTR2, SSTR3, SSTR4 and SSTR5) (reviewed by Law, Woulfe & Reisine, 1995). Among these SRIF receptor subtypes, SSTR2, SSTR3 and SSTR5 are coupled to PTX-sensitive G proteins (Law *et al.* 1995). Using *in situ* hybridization, Pérez *et al.* (1994) investigated the distribution of SRIF receptors in the rat brain and reported that locus coeruleus neurones expressed mRNAs of SSTR2 and SSTR3, but not of SSTR1. (However, Breder, Yamada, Yasuda, Seino, Saper & Bell (1992) observed that locus coeruleus neurones from the mouse brain expressed SSTR1 but not SSTR2 mRNA.) Reisine & Bell (1995) reported that there are rat brain SRIF receptors which are different from the five already cloned SRIF receptors. Thus, the involved SRIF receptor subtype in our experiments on rat locus coeruleus neurones is either SSTR2 or SSTR3, or an as yet unidentified SRIF receptor. Coupling between SRIF receptors and G proteins was investigated, using immunoprecipitation in the absence of agonist, by Law, Manning & Reisine (1991). They found that endogenous rat brain SRIF receptors coupled to $G\alpha_{i1}$ and $G\alpha_{i3}$, but not to $G\alpha_{i2}$. On the other hand, in the presence of agonist, SRIF receptors were found to couple to $G\alpha_{i2}$ (Law & Reisine, 1992; Murray-Whelan & Schlegel, 1992; Luthin, Eppler & Linden, 1993). These biochemical data and the present physiological results suggest that the SRIF receptor subtype which modulates the inward rectifier in locus coeruleus neurones couples to G_{i2}, and that the coupling occurs only when activated by agonist.

SRIF response in AtT-20 cells

AtT-20 cells are known to possess PTX-sensitive G proteins (G_{i1}, G_{i2}, G_{i3} and G_o) (Law *et al.* 1991). The cells also express multiple subtypes of SRIF receptors: SSTR2 (most abundant), SSTR5, SSTR4 and SSTR1 (least abundant) (Patel, Panetta, Escher, Greenwood & Srikant, 1994).

The present study using antibodies indicates that $G\alpha_{i3}$ is involved in the SRIF-induced activation of the inward rectifier K^+ current in AtT-20 cells. The involvement of G_{i3} in the SRIF-induced activation of an inward rectifier was also observed in another endocrine cell type, growth hormone-secreting human pituitary cells in primary culture (Takano, Yasufuku-Takano, Teramoto & Fugita, 1997). In contrast, SRIF modulates adenylyl cyclase through $G\alpha_{i1}$ in AtT-20 cells (Tallent & Reisine, 1992). These results indicate that in AtT-20 cells SRIF exerts its effects on two different effectors through two different types of G proteins; G_{i1} mediates the modulation of adenylyl cyclase activity, and G_{i3} activates the $K_{IR}(G)$ channels.

In our experiments on AtT-20 cells, unlike those on locus coeruleus neurones, we could not determine the role of G_o in the activation of the inward rectifier because the only available antibody against the C-terminus of $G\alpha_o$ is anti-$G\alpha_o/G\alpha_{i3}$, and this antibody cannot differentiate the involvement of $G\alpha_o$ from that of $G\alpha_{i3}$. Furthermore, either because the cells were fragile or because the cells divide, we were unable to do antisense experiments. G_o is known to mediate Ca^{2+} current inhibition by SRIF in endocrine cells (Kleuss *et al.* 1991; Chen & Clarke, 1996).

The subtype of SRIF receptor that mediates the activation of the inward rectifier K^+ channel in AtT-20 cells is unknown. Using selective agonists to SRIF receptor subtypes, it was found that SSTR2 and SSTR5 mediated the inhibition of voltage-gated L-type Ca^{2+} channels in AtT-20 cells (Tallent, Dichter & Reisine, 1996*a*), whereas the SRIF receptor subtype involved in the activation of the inward rectifier K^+ channel was not one of the known cloned SRIF receptors (Tallent, Dichter & Reisine, 1996*b*). In the absence of agonist, SRIF receptors immunoprecipitate with G_{i1}, G_{i3} and G_o (Law *et al.* 1991) in AtT-20 cells. This coupling differs from the coupling between SRIF receptors and G_{i2}, which was observed only in the presence of agonist (Law & Reisine, 1992). Thus, the results of the present experiments, combined with the past literature, suggest that in AtT-20 cells a still unknown subtype of SRIF receptor and G_{i3} mediate the activation of inward rectifier K^+ currents.

Specificity of G protein α-subunits

As mentioned above, we found that the activation of the $K_{IR}(G)$ current is mediated through different types of G proteins in neurones and in endocrine cells: G_{i2} in locus coeruleus neurones and G_{i3} in AtT-20 cells. This result, however, does not necessarily mean that the $K_{IR}(G)$ channels in locus coeruleus neurones are directly activated by $G\alpha_{i2}$ or that those in AtT-20 cells are directly activated by $G\alpha_{i3}$. On the contrary, evidence has accumulated indicating that the inward rectifier is activated by the $\beta\gamma$-subunits rather than the α-subunit of the G protein. In endogenous and in cloned atrial G protein-coupled inward rectifiers (GIRKs), it has been shown that the G protein $\beta\gamma$-subunits, not the α-subunit, activate the channels (Reuveny *et al.* 1994; Wickman *et al.* 1994). Furthermore, in atrial cells different types of $\beta\gamma$-subunits are capable of activating the atrial GIRKs equally well (Wickman *et al.* 1994). We obtained evidence that in locus coeruleus neurones G protein $\beta_1\gamma_2$ subunits (not α_{i2} subunits) directly activate the inward rectifier K^+ channel (Nakajima, Nakajima & Kozasa, 1996). Thus, the G protein specificity that we observed in the SRIF-induced activation of the inward rectifier probably resides in the coupling of SRIF receptors and specific G proteins (α-subunits).

Still, if the non-specificity of $\beta\gamma$-subunits holds true, ambiguity remains. In AtT-20 cells, Tallent *et al.* (1996*a*) reported that two SRIF receptor subtypes, SSTR2 and SSTR5, mediate the inhibition of voltage-gated Ca^{2+} channels. In GH_3 cells (another cell line from the anterior pituitary), SRIF-induced inhibition of Ca^{2+} currents is mediated by G_o (Kleuss *et al.* 1991). In ovine somatotrophs (Chen & Clarke, 1996) and in AtT-20 cells (Law & Reisine, 1992) SRIF receptors couple to G_o. Further, G_{i1} couples to adenylyl cyclase (Tallent & Reisine, 1992). These reports, as well as the present data, indicate that more than one type of G protein couples to SRIF receptors within one cell. If most $\beta\gamma$-subunits are capable of activating the inward rectifier non-specifically, how does the specificity arise? In the example of AtT-20 cells, why were only the $\beta\gamma$-subunits that were coupled to $G\alpha_{i3}$ able to activate the inward rectifier, while the $\beta\gamma$-subunits coupled to $G\alpha_{i1}$ (which modulates adenylyl cyclase) were unable to activate the inward rectifier? Recently, Huang, Slesinger, Casey, Jan & Jan (1995) and Slesinger, Reuveny, Jan & Jan (1995) proposed a theory which could answer this question. They presented data suggesting that the three components (receptor, G protein, and GIRK) may form a complex under physiological conditions, and because of the physical proximity of these components only a certain type of G protein may be able to activate the inward rectifier. The specificity of the coupling between SRIF receptor and G protein, combined with constraints resulting from their physical proximity, could explain the specificity of the G protein coupling that we observed in the present experiments.

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