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ACCELERATED COMMUNICATION

$G_{i\alpha 1}$ Selectively Couples Somatostatin Receptors to Adenylyl Cyclase in Pituitary-Derived AtT-20 Cells

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SUMMARY

Somatostatin (SRIF) receptors are coupled to the catalytic subunit of adenylyl cyclase via pertussis toxin-sensitive guanine nucleotide-binding regulatory proteins (G proteins). To identify which G proteins link SRIF receptors to adenylyl cyclase, $G_{o\alpha}$, $G_{i\alpha}$, and its different subtypes were individually blocked in AtT-20 cell membranes with G_α subtype-selective antisera. Antiserum directed against the carboxyl-terminal region of $G_{i\alpha}$ blocked SRIF inhibition of forskolin-stimulated adenylyl cyclase activity, and this effect was prevented by the peptide to which the antiserum was generated. However, antiserum directed against the carboxyl-terminal region of $G_{o\alpha}$ did not affect SRIF inhibition of adenylyl cyclase activity, indicating that $G_{i\alpha}$ couples SRIF receptors to adenylyl cyclase but $G_{o\alpha}$ does not. Peptide-directed antisera against $G_{i\alpha 1}$ completely blocked SRIF inhibition of adenylyl cyclase activity. In contrast, antisera directed against either $G_{i\alpha 2}$ or $G_{i\alpha 3}$ did not affect the actions of SRIF. The results of these studies indicate that $G_{i\alpha 1}$ selectively couples SRIF receptors to the catalytic subunit of adenylyl cyclase in AtT-20 cell membranes. Because previous studies have shown that SRIF receptors are able to couple to $G_{i\alpha 1}$, $G_{i\alpha 3}$, and $G_{o\alpha}$, the results suggest that different G proteins may specify the coupling of SRIF receptors to distinct cellular effector systems.

The neuropeptide SRIF inhibits hormone release from the pituitary and pancreas and modulates neuronal firing activity in both the central and peripheral nervous system (1). The cellular actions of SRIF are mediated by membrane-bound receptors. Activation of these receptors leads to inhibition of adenylyl cyclase activity (2, 3) and Ca^{2+} conductance (4, 5), as well as the potentiation of K^+ currents (6, 7). SRIF receptors are coupled to the catalytic subunit of adenylyl cyclase and ionic conductance channels via pertussis toxin-sensitive G proteins (8). The results of recent immunoprecipitation studies (9) have shown that particular α subunits of G_i and G_o couple to SRIF receptors from rat brain and the pituitary cell line AtT-20. Selectivity exists in the subtypes of $G_{i\alpha}$ that couple with the SRIF receptor, because $G_{i\alpha 1}$ and $G_{i\alpha 3}$ are associated with the receptor but $G_{i\alpha 2}$ is not, suggesting that SRIF receptors are linked to cellular effector systems via $G_{i\alpha 1}$, $G_{i\alpha 3}$, and $G_{o\alpha}$.

In the present study, we have investigated whether selectivity exists in the G protein subtype linking the SRIF receptor to adenylyl cyclase in AtT-20 cells. The approach employed is similar to that used by Simmonds *et al.* (10) and McKenzie and Milligan (11), who showed that antibodies directed against $G_{i\alpha 2}$ could selectively block α_2-adrenergic and opiate inhibition of adenylyl cyclase activity in human platelet and NG-108 cell membranes, whereas antisera against the other pertussis toxin-sensitive α subunits did not. Our findings indicate the $G_{i\alpha 1}$ selectively couples SRIF receptors to adenylyl cyclase in AtT-20 cells, supporting the hypothesis that individual G proteins associated with the SRIF receptor may couple the receptor to different cellular effector systems.

Experimental Procedures

Materials. [^{32}P]ATP and [^{3}H]cAMP were obtained from NEN-DuPont (Albany, NY). SRIF-28 was purchased from Bachem (Torrance, CA). Forskolin was obtained from Calbiochem (San Diego, CA). All other biochemicals were obtained from Boerhinger-Mannheim (Indianapolis, IN).

Antisera. Anti-G_α antisera were provided by Dr. David R. Manning (University of Pennsylvania) and have been previously described and characterized (9, 12, 13). The G_α-directed antisera were all obtained after injection of synthetic peptides, corresponding to specific regions of G_α into rabbits. Antiserum 8730 is directed against a carboxyl-terminal region of $G_{i\alpha}$ (KNNLKDCGLF; residues 345–354 of $G_{i\alpha 1}$, 346–355 of $G_{i\alpha 2}$) and selectively detects and immunoprecipitates $G_{i\alpha 1}$, $G_{i\alpha 2}$,

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ABBREVIATIONS: SRIF, somatostatin; G protein, guanine nucleotide-binding regulatory protein; G_i, inhibitory guanine nucleotide-binding protein; G_o, predominant pertussis toxin-sensitive guanine nucleotide-binding protein expressed in brain; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid.

and to a lesser extent $G_{i\alpha 3}$ (9, 12). Antisera 3646 (anti-$G_{i\alpha 1}$) (CLDRIAQPNYI; residues 159–167) and 1521 (anti-$G_{i\alpha 2}$ (CLERIAQSDYI; residues 160–168) were made against the peptides corresponding to the same internal region of $G_{i\alpha}$ that is divergent in sequence for $G_{i\alpha 1}$ and $G_{i\alpha 2}$. The antisera are selective in detecting recombinant $G_{i\alpha}$ subtypes by Western blotting (13) and immunoprecipitate $G_{i\alpha}$ subtypes equally well (9). Antiserum 1518 (anti-$G_{i\alpha 3}$) (CIDFGEAARADDAR; residues 93–105) was generated against an internal region of $G_{i\alpha 3}$ and specifically interacts with $G_{i\alpha 3}$ (13). Antiserum 9072 (anti-$G_{o\alpha}$) (ANNLRGCGLY; residues 345–354) is directed against the same region of G_{α} as 8730 that differs in sequence between $G_{o\alpha}$ and $G_{i\alpha}$. It selectively detects recombinant forms of $G_{o\alpha}$ by Western blotting[1] and effectively immunoprecipitates $G_{o\alpha}$.[2]

Cell culture techniques. Mouse AtT-20 cells were grown and subcultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, as previously described (3). Cells were grown in 75-cm^2 culture flasks at an initial density of 1×10^6 cells/flask and were used 5 or 6 days after subculturing (approximately 80% confluent).

Membrane preparation. AtT-20 cell membranes were prepared as previously described (3). Briefly, cells were harvested with 5 ml of buffer containing 50 mM Tris·HCl (pH 7.4) containing 1 mM EGTA, 5 mM $MgCl_2$, 10 μg/ml leupeptin, 10 μg/ml pepstatin, 200 μg/ml bacitracin, and 0.5 μg/ml aprotinin (buffer 1). The cells were centrifuged for 10 min at 20,000 × *g*, and the pellet was homogenized in 1 ml of buffer 1, using a Brinkman homogenizer (setting 2, 10 sec). Four milliliters of buffer 1 were added, and the cells were centrifuged at 1400 × *g* for 5 min. The supernatant was removed and centrifuged at 35,000 × *g* for 30 min. The pellet was homogenized and diluted to 100–200 μg/ml in buffer 1.

Adenylyl cyclase assay. Membranes (360 μl) were incubated with antisera (1/40 dilution) at 4° for 1 hr before the assay. In studies involving peptide blockade of the actions of the antisera, the antiserum was preincubated with its peptide (40 μg) for 1 hr at 4° before being added to the membranes. Adenylyl cyclase activity was measured by adding 20 μl of membrane (2–4 μg of protein) to 80 μl of an incubation solution containing 50 mM Tris·HCl (pH 7.8), 50 μM ATP, 0.1 mM cAMP, 30 μM GTP, 2 mM $MgCl_2$, 100 mM NaCl, 0.5 mM isobutylmethylxanthine, 5 mM phosphocreatine, 40 μg of creatine phosphokinase, and 1–2 × 10^6 cpm of [^{32}P]ATP, with or without 50 μM forskolin and/or 10 μM SRIF-28. The reaction was carried out for 6 min and was terminated by the addition of 100 μl of 10% sodium dodecyl sulfate, 5 mM ATP, in 50 mM Tris·HCl (pH 7.4). [^{3}H]cAMP was added (5000 cpm) to monitor recovery through the separation procedure. The amount of ^{32}P-labeled cAMP formed from [^{32}P]ATP was determined by the method of Salomon *et al.* (14).

Results

AtT-20 cells express a high density of SRIF receptors that are coupled to adenylyl cyclase via pertussis toxin-sensitive G proteins (3, 8, 9). The cells have been previously shown to express $G_{i\alpha 1}$, $G_{i\alpha 2}$, $G_{i\alpha 3}$, and $G_{o\alpha}$ (9). To further demonstrate the presence of G protein α subunits in AtT-20 cell membranes, the G protein α subunits in AtT-20 cells were detected by immunoblotting, using the peptide-directed antisera against $G_{i\alpha}$ (8730), $G_{o\alpha}$ (9072), $G_{i\alpha 1}$ (3646), $G_{i\alpha 2}$ (1521), and $G_{i\alpha 3}$ (1518) (Fig. 1). All five antisera were able to detect proteins migrating in the size range of 39–41 kDa. Furthermore, these antisera were previously reported to immunoprecipitate similar levels of pertussis toxin-sensitive G proteins from solubilized AtT-20 cell proteins (9). The selectivity of the antisera has been demonstrated based on their specific interaction with recombinant forms of G protein α subunits (13).

[1] D. Manning, personal communication.
[2] S. Law and T. Reisine, unpublished observations.

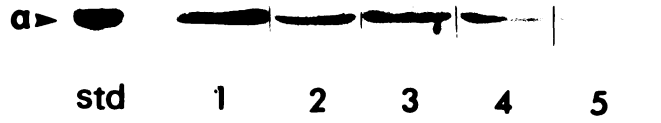

Fig. 1. Detection of G protein α subunits in AtT-20 cell membranes, by immunoblotting. Proteins from AtT-20 cell membranes and purified $G_{i\alpha}$ from rat liver were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunoblotting using antisera 8730 (anti-$G_{i\alpha}$ common) (*lane 1*), 9072 (anti-$G_{o\alpha}$) (*lane 2*), 3646 (anti-$G_{i\alpha 1}$) (*lane 3*), 1521 (anti-$G_{i\alpha 2}$) (*lane 4*), and 1518 (anti-$G_{i\alpha 3}$) (*lane 5*), as previously described (9). The purified $G_{i\alpha}$ was reacted with 8730. Antisera were used at a dilution of 1/500 for immunoblotting. Equal amounts of proteins from AtT-20 cells were added to each lane.

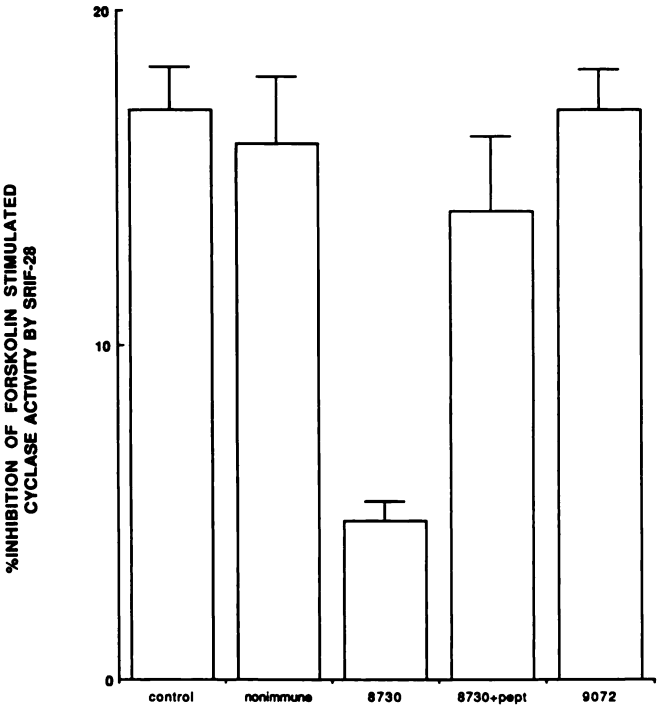

Fig. 2. Effect of $G_{i\alpha}$ and $G_{o\alpha}$ antisera on the inhibition of forskolin-stimulated adenylyl cyclase activity by SRIF-28. Forskolin-stimulated adenylyl cyclase activity was maximally inhibited by 10 μM SRIF-28 (seven experiments) (in representative experiments, basal activity = 73.5 ± 5.9, forskolin-stimulated = 206 ± 17, and forskolin plus SRIF-28 = 166 ± 17 pmol/mg/min) (*control*). Nonimmune sera (four experiments) and antiserum 9027 (five experiments) did not affect SRIF-28 inhibition of adenylyl cyclase activity. Antiserum 8730 (seven experiments) blocked the inhibition of forskolin-stimulated adenylyl cyclase activity by SRIF-28 ($p < 0.05$, Student's *t* test), and this effect was prevented by preincubating antiserum 8730 with 40 μg of the peptide to which it was generated (*8730+pept*) (four experiments). Values are presented as the mean ± standard error of percentage of inhibition of forskolin-stimulated adenylyl cyclase by SRIF-28.

To determine which G proteins couple SRIF receptors to adenylyl cyclase in AtT-20 cell membranes, we have selectively blocked different G protein α subunits with peptide-directed antisera and studied the ability of the potent agonist SRIF-28 to inhibit forskolin-stimulated adenylyl cyclase activity. In control membranes, SRIF-28 inhibited forskolin-stimulated adenylyl cyclase activity by 18 ± 1% (Fig. 2). Nonimmune serum did not affect the ability of SRIF-28 to inhibit forskolin-stimulated adenylyl cyclase activity (Fig. 2). However, antiserum 8730, which is directed against the carboxyl-terminal region of $G_{i\alpha}$ and interacts with all three forms of $G_{i\alpha}$, blocked SRIF-28 inhibition of forskolin-stimulated adenylyl cyclase activity (Fig. 2). This effect was prevented by the peptide to

which antiserum 8730 was generated (Fig. 2). The ability of 8730 to block the effect of SRIF-28 on adenylyl cyclase activity was maximal at the dilution of antiserum used, based on the results of saturation studies (data not shown). Antiserum 8730 did not alter basal or forskolin-stimulated adenylyl cyclase activity under these conditions. Because previous studies (9) have shown that antiserum 8730 does not prevent the interaction of SRIF agonists with the receptor or uncoupled SRIF receptors from $G_{i\alpha}$, the results of the present study indicate that antiserum 8730 prevents the association of the SRIF receptor/$G_{i\alpha}$ complex with the catalytic subunit of adenylyl cyclase.

In contrast, antiserum 9072, which is directed against the carboxyl-terminal of $G_{o\alpha}$, did not affect SRIF-28 inhibition of adenylyl cyclase activity (Fig. 2). Antisera 9072 and 8730 are directed against the same region of $G_{i\alpha}$ that differs in sequence in $G_{o\alpha}$ and $G_{i\alpha}$, suggesting that the lack of effect of antiserum 9072 cannot be due to epitope inaccessibility. Previous studies have shown that SRIF receptors in AtT-20 cells associated with $G_{o\alpha}$ (9), and antiserum 9072 can immunoprecipitate $G_{o\alpha}$ and SRIF receptor/$G_{o\alpha}$ complexes from AtT-20 cells, indicating that the antiserum can interact with native $G_{o\alpha}$ and the SRIF receptor/$G_{o\alpha}$ complex.[2] These findings indicate that, although both $G_{i\alpha}$ and $G_{o\alpha}$ are coupled to SRIF receptors, only $G_{i\alpha}$ links the receptor to adenylyl cyclase.

Three subtypes of $G_{i\alpha}$ are expressed in AtT-20 cells. Antiserum 3646 selectively interacted with $G_{i\alpha1}$ and abolished SRIF-28 inhibition of forskolin-stimulated adenylyl cyclase activity (Fig. 3). The dilution of antiserum 3646 used maximally blocked SRIF-28 inhibition of adenylyl cyclase activity, based on the results of saturation studies (data not shown). Antiserum 3646 did not affect basal or forskolin-stimulated adenylyl cyclase activity under these conditions. Because previous studies (9) have shown that antiserum 3646 does not interfere with the interaction of SRIF agonists with its receptor or uncouple the receptor from G proteins, the present results indicate that antiserum 3646 prevents the association of SRIF receptor/$G_{i\alpha1}$ complexes with the catalytic subunit of adenylyl cyclase.

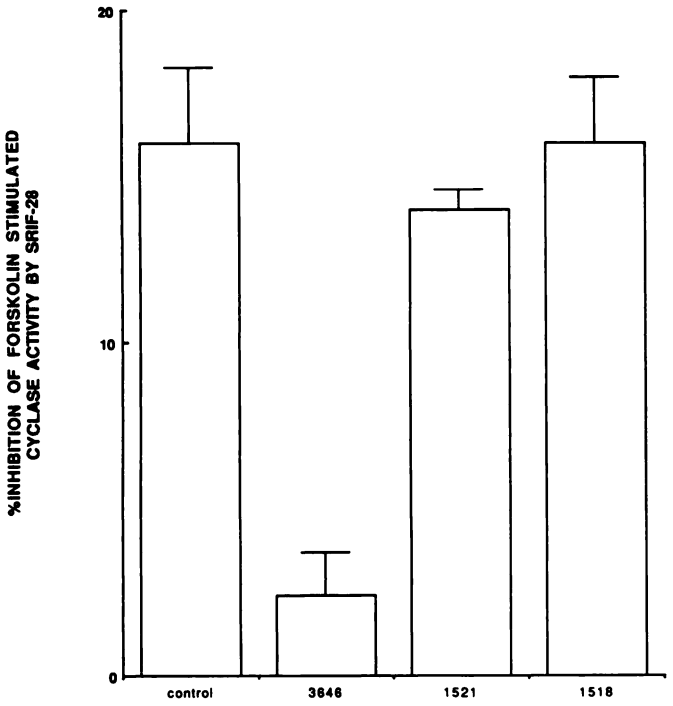

Fig. 3. Antiserum directed against $G_{i\alpha1}$ selectively prevents SRIF-28 from inhibiting forskolin-stimulated adenylyl cyclase activity. The ability of antisera 3646 (anti-$G_{i\alpha1}$), 1521 (anti-$G_{i\alpha2}$), and 1518 (anti-$G_{i\alpha3}$) to prevent the inhibition of forskolin-stimulated adenylyl cyclase activity by SRIF-28 (10 μM) was tested. Values presented are the mean ± standard error of percentage of inhibition of forskolin-stimulated adenylyl cyclase activity of five different experiments done in quadriplicate. The blockade of SRIF-28 inhibition of forskolin-stimulated adenylyl cyclase activity by antiserum 3646 was statistically significant ($p < 0.05$, using a Student's t test).

In contrast to the effects of antiserum 3646, antisera 1521 and 1518, which selectively interact with $G_{i\alpha2}$ and $G_{i\alpha3}$, respectively, did not affect SRIF-28 inhibition of forskolin-stimulated adenylyl cyclase activity (Fig. 3). Antisera 1521 and 3646 are directed against the same region of $G_{i\alpha}$ that differs in amino acid sequence between $G_{i\alpha2}$ and $G_{i\alpha1}$ indicating that the lack of effect of antiserum 1521 cannot be due to variations in epitope accessibility. In addition, antiserum 1521 has been shown to immunoprecipitate $G_{i\alpha}$ (9), indicating that it is able to recognize native $G_{i\alpha2}$. Furthermore, previous studies (9) have shown that antiserum 1518 can immunoprecipitate solubilized $G_{i\alpha}$ and SRIF receptor/$G_{i\alpha3}$ complexes from AtT-20 cells, indicating that its lack of effect on the actions of SRIF-28 are not due to its inability to associate with native $G_{i\alpha3}$ or SRIF receptor/$G_{i\alpha3}$ complexes. The results of these studies indicate that $G_{i\alpha1}$ selectively couples SRIF receptors to adenylyl cyclase in AtT-20 cell membranes.

Discussion

Our previous studies (9), using an immunoprecipitation approach to identify the G proteins coupled to SRIF receptors, indicated that SRIF receptors associate with $G_{i\alpha1}$, $G_{i\alpha3}$, and $G_{o\alpha}$ in AtT-20 cells. The results of our present study suggest that $G_{i\alpha1}$ selectively couples the AtT-20 cell SRIF receptor to adenylyl cyclase. These findings are consistent with the results of recent studies suggesting that different G proteins couple SRIF receptors to distinct cellular effector systems. Kleuss *et al.* (15), using an "mRNA knock-out" procedure to block the expression of α subunits of G proteins in GH_3 cells, reported that $G_{o\alpha2}$ selectively couples SRIF receptors to Ca^{2+} channels. Furthermore, reconstitution studies by Yatani *et al.* (16) have provided indirect evidence that $G_{i\alpha3}$ (G_k) may couple SRIF receptor to K^+ channels in GH_3 cell patches. Taken together, these findings create a simple model to explain the molecular basis of the cellular actions of SRIF in which the SRIF receptor couples to three different α subunits and each α subunit links the receptor to a distinct cellular effector system.

The ability of SRIF receptors in AtT-20 cells to couple to three distinct G proteins that link the receptor to different cellular effector systems suggests that G proteins contribute to the functional diversity of SRIF receptors. This model system suggests that $G_{i\alpha1}$, $G_{i\alpha3}$, and $G_{o\alpha}$ may compete for common recognition sites in the SRIF receptor. Such competition may be critical in determining which cellular effector system is modulated after the binding of SRIF to its receptor.

Different G proteins have also been reported to diversify α_2-adrenergic receptor function. Simmonds *et al.* (10) reported that $G_{i\alpha2}$ selectively couples α_2-adrenergic receptors to adenylyl cyclase in platelet membranes, whereas McFadzean *et al.* (17) have reported that $G_{o\alpha}$ selectively couples α_2 receptors to Ca^{2+} channels in NG-108 cells. These findings suggest that different G proteins couple α_2 receptors to distinct cellular effector

systems. Furthermore, different G proteins have been reported to link muscarinic receptors to distinct cellular effector systems (18), and the findings of Matesic *et al.* (19, 20), using biochemical and immunoprecipitation approaches, confirm that muscarinic receptors are capable of associating with multiple G proteins. In fact, a number of neurotransmitter receptors, including adenosine (21) and dopamine$_2$ (22) receptors, have been found to interact with more than one α subunit subtype. Taken together, these findings suggest that G proteins may contribute to the complexity of cellular actions regulated by neurotransmitters, by linking individual receptors to distinct cellular effector systems.

Recent studies have shown that subtypes of SRIF receptors are expressed in mammalian cells (23, 24). Two subtypes ($SRIF_1$ and $SRIF_2$ receptors) can be distinguished by their sensitivities to the peptide MK 678. Although both SRIF receptor subtypes have been shown to mediate SRIF inhibition of adenylyl cyclase activity (24, 25), no information is available on whether they couple to similar G proteins or whether the same G protein couples both receptors to adenylyl cyclase. AtT-20 cells predominantly express $SRIF_1$ receptors,[3] and our present findings indicate that $G_{i\alpha1}$ is the predominant G protein coupling this receptor to adenylyl cyclase. In S49 lymphoma cells, SRIF receptors are expressed and have been reported to mediate SRIF inhibition of adenylyl cyclase activity in S49 lymphoma cells (26). Yet, studies by Mumby *et al.* (27) suggest that relatively little $G_{i\alpha1}$ immunoreactivity can be detected in S49 lymphoma cell membranes with a peptide-directed antiserum (I-355) against $G_{i\alpha1}$. These findings indicate that G proteins other than $G_{i\alpha1}$ may mediate SRIF inhibition of adenylyl cyclase activity in S49 lymphoma cells. Studies are underway to investigate whether S49 lymphoma cells express SRIF receptors different from those in AtT-20 cells and whether these receptors couple to adenylyl cyclase via different G proteins than $SRIF_1$ receptors. Such studies may establish whether SRIF receptor subtypes couple to different G proteins.

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[3] K. Raynor and T. Reisine, unpublished observations.

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