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Pituitary Cell Line GH_3 Expresses Two Somatostatin Receptor Subtypes that Inhibit Adenylyl Cyclase: Functional Expression of Rat Somatostatin Receptor Subtypes 1 and 2 in Human Embryonic Kidney 293 Cells

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SUMMARY

Using a polymerase chain reaction approach, we have studied the expression of somatostatin receptor (SSTR) subtypes in the GH_3 rat pituitary cell line, a well established *in vitro* model for the cellular effects of somatostatin. We found that the previously identified SSTR1 and SSTR2 are the major subtypes expressed in this cell line. No other SSTR subtype was detected by our analysis. Northern blots confirmed that both subtypes, but not SSTR3, are expressed in GH_3 cells. We studied the functional expression of both SSTR subtypes by transfection of their cDNAs into human embryonic kidney 293 cells. We found that somatostatin inhibited cAMP accumulation in human embryonic kidney 293 cells only when cells were transfected with either SSTR1 or SSTR2. This inhibition was blocked by treatment of the transfected cells with pertussis toxin, demonstrating that it is mediated by G proteins sensitive to this toxin. In addition, we provide pharmacological evidence that the endogenous SSTR2 subtype mediates inhibition of cAMP accumulation in intact GH_3 cells. Our results contradict previous reports that concluded that neither SSTR1 nor SSTR2 is involved in inhibition of adenylyl cyclase. The reasons for this apparent contradiction are discussed. We conclude that both SSTR1 and SSTR2 are capable of coupling to pertussis toxin-sensitive G proteins to inhibit adenylyl cyclase.

Somatostatin is a 14-amino acid peptide that has many physiological effects on endocrine (1) and nerve (2) cells. In endocrine cells, the primary effect of the peptide is the inhibition of secretion of several hormones (3–9). This peptide exerts its effects through cell surface receptors that appear to be heterogeneous (10). Receptor activation triggers a series of effects on intracellular second messengers, such as the inhibition of adenylyl cyclase (11, 12), inhibition of voltage-dependent calcium currents (13–15), stimulation of potassium channels (16–18), and regulation of sodium/proton exchange (19). The concerted action of these second messengers results in the inhibition of secretion of hormones by endocrine cells in response to somatostatin.

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Somatostatin was identified and isolated because of its ability to inhibit growth hormone secretion from the anterior pituitary (3). This effect of somatostatin has been preserved in the rat pituitary cell line GH_3 (20, 21), which has been extensively used as a model system to study the mechanisms that regulate hormone secretion, and in particular the effects of somatostatin. Recently, several research groups (22–28) reported the cloning of cDNAs encoding different subtypes of SSTRs. In the present work, we have determined that two SSTR subtypes (SSTR1 and SSTR2) are expressed in the GH_3 cell line. No other SSTR subtype was detected in our search, indicating that SSTR1 and SSTR2 are the major SSTR subtypes expressed in GH_3 cells. Surprisingly, it has been recently reported that neither of these SSTR subtypes mediates inhibition of adenylyl cyclase (10, 25, 29), an effect of somatostatin that has been well established in GH_3 cells (12). This observation raises the question of the mechanism by which somatostatin inhibits adenylyl cyclase in GH_3 cells. We have reexamined this question by introducing cDNAs for both

ABBREVIATIONS: SSTR, somatostatin receptor; (*R*)-PIA, (−)-N^6-(2-phenylisopropyl)adenosine; PCR, polymerase chain reaction; SDS, sodium dodecyl sulfate; SSC, standard saline citrate; PTX, pertussis toxin; DME, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; MEM, minimum essential medium; kb, kilobase(s); CHO, Chinese hamster ovary; HEK, Human embryonic kidney; HEPES, 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid.

SSTR1 and SSTR2 (isolated from a GH_3 cells cDNA library) into HEK 293 cells and determining the effects of somatostatin on cAMP accumulation.

Experimental Procedures

Materials. Oligonucleotides were synthesized in a PCR-Mate 391 DNA synthesizer (Applied Biosystems, Foster City, CA). Radioisotopes and Hybond-N^+ nylon membranes were purchased from Amersham (Arlington Heights, IL). Vasoactive intestinal peptide and somatostatin were purchased from Peninsula Laboratories (Belmont, CA). MK-678 was a generous gift of Dr. D. Viber, Merck Sharp & Dohme Research Laboratories (West Point, PA). Dopamine and (*R*)-PIA were purchased from Sigma Chemical Co. (St. Louis, MO). All other chemicals were purchased from Fisher Scientific (Pittsburgh, PA) or Sigma Chemical Co. (St. Louis, MO). The human dopamine D1 receptor is identical to that reported by Zhou *et al.* (30), cloned into the expression vector pCDM7. The rat adenosine A1 receptor was cloned from the same GH_3 cDNA library described below.[3]

Screening of a GH_3 cDNA library for SSTR subtypes. The screening of the cDNA library was performed by using the PCR technique to detect SSTR cDNAs in pools of cDNA clones. DNA from a GH_3 cDNA library (constructed in the expression vector pCDM7) was transfected into the *Escherichia coli* strain MC1061/P3, and cells were plated at a density of 5000 clones/plate. The colonies were then scraped from the plate and mixed homogeneously. The cell suspension was used to inoculate liquid cultures for plasmid DNA preparation by the alkaline-SDS method (31). DNA from 96 pools was prepared, digested with *Xho*I (which cut out the cDNA insert from the plasmid vector), resolved by electrophoresis in 1% agarose gels, and blotted onto Hybond-N^+ nylon membranes (31). DNA from these pools was also used as template in PCRs to obtain specific DNA probes for the SSTRs present in the GH_3 cDNA library. Two degenerate oligonucleotides were synthesized that could amplify all of the known SSTR subtypes (22–26). These oligonucleotides corresponded to the conserved sequences in transmembrane region II [5′-AA(CT)CT(GCT)GC(CT)(CA)T(GCT)GC(CT)GA(CT)GA-3′] and transmembrane region VII [5′-(GA)GG(GA)TT(GA)GC(GA)CA(GA)(GC)(TA)(GA)TT(GA)GC(AG)TA-3′] (22–26). PCR assays with these primers and DNA from the first 20 cDNA pools resulted in specific products from 14 of the pools. These PCR products were labeled by random priming (31) and used as probes in high stringency hybridizations (hybridization at 65° in 6× SSC (1 × SSC is 0.15 M NaCl and 0.015 M Sodium Citrate), 1% SDS, 1% skim milk powder; washes at 65° in 0.1× SSC, 0.1% SDS) with Southern blots of the 96 cDNA pools. Two distinct hybridization patterns for the 96 pools were detected with the 14 probes, indicating the presence of two SSTR subtypes. The DNA from all pools that were negative in the Southern blot analysis was used as the template in similar PCRs. This experiment failed to detect any other SSTR subtype in the cDNA library. Most pools contained a single cDNA isolate for each SSTR subtype, as judged by the number of hybridization bands on the Southern blots. However, a few pools contained both SSTR subtypes. The differences in the size of the hybridization bands from pool to pool indicated that several distinct clones for each SSTR subtype were present in the cDNA library. Pools were selected for isolation of the respective SSTR clones on the basis of the cDNA insert size. The sequences of the coding regions of these clones were determined, demonstrating that they correspond to SSTR1 and SSTR2 (data not shown).

Northern blot analysis. Total RNA from GH_3 and RINm5F cells in 10 nearly confluent, 10-cm, tissue culture plates and RNA from 100 rat pituitaries were extracted by the guanidinium thiocyanate method (31). Poly(A)$^+$ RNA, selected as described (31), was electrophoresed in 1% agarose-formaldehyde gels and transferred to Hybond-N^+ membranes as described (31). Hybridizations were performed at 42° for 16–20 hr in 6× SSC, 44% formamide, 10% dextran sulfate, 0.8% SDS, 1× Denhardt's solution, 0.1 mg/ml denatured salmon sperm DNA. After hybridization, the blots were washed two times, for 30 min each time, at 55° with 2× SSC/0.1% SDS and two times, for 30 min each time, at 55° with 0.1× SSC/0.1% SDS. After the washes, the blots were exposed to Kodak X-Omat film as indicated in the legend to Fig. 1. The DNA probes used for these hybridizations were generated by PCR to avoid contamination with plasmid DNA, which binds nonspecifically to residual rRNA present in the poly(A)$^+$ RNA.

Tissue culture. All tissue culture materials were obtained from the University of California, San Francisco, Tissue Culture Facility. GH_3 and RINm5F cells were maintained in DME-H21 medium supplemented with 10% FCS. HEK 293 cells were maintained in Eagle's MEM supplemented with 10% horse serum.

Transfection of HEK 293 cells. HEK 293 cells (CRL 1573; purchased from the American Type Culture Collection) were plated in Eagle's MEM supplemented with 10% FCS, at a density of 5×10^6 cells/10-cm culture plate, the day before transfection. The plasmid DNA mixtures were diluted to concentrations below 0.5 mg/ml before mixing with 5 ml of Eagle's MEM containing 2.5% FCS, 0.4 mg/ml DEAE-dextran, and 20 μM chloroquine phosphate. The culture medium was removed from the culture plates before application of the 5-ml transfection mixture. After a 2-hr incubation, the transfection mixture was removed and the cells were shocked for 2 min with phosphate-buffered saline containing 10% dimethylsulfoxide. This solution was removed and the cells were incubated for 24–30 hr in Eagle's MEM containing 10% FCS. The transfected cells from one 10-cm culture plate were removed by trypsin treatment and divided as follows: 90% of the cells were divided into the wells of a 24-well culture plate for the cAMP accumulation assay (see below) and the remaining 10% were transferred to a 6-cm culture plate to test the transfection efficiency. The latter control was performed in all of the experiments described in this paper. Transfection efficiency was monitored by including in the plasmid mixture (in addition to the expression vectors indicated for each experiment) a vector containing the *E. coli* β-galactosidase gene under the control of the cytomegalovirus promoter (pON249). The transfection efficiency was determined as the percentage of cells that expressed β-galactosidase 48 hr after transfection. This was determined by staining the cells with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside. Typically 5–10% of the cells were transfected under the conditions described, and occasionally we obtained higher efficiencies (the highest that we observed was about 20%). Cells transfected at efficiencies of <1% were discarded.

Assay for cAMP accumulation. This assay is a modification of the method of Salomon *et al.* (32) and has been previously described by Federman *et al.* (33). Briefly, 24 hr after transfer into 24-well plates the culture medium was removed and replaced with 0.5 ml/well of labeling medium (DME-H21 containing no sodium bicarbonate and supplemented with 20 mM HEPES, 10% FCS, and 2 μCi/ml [^{3}H] adenine). The cells were then incubated for 12–16 hr before treatment with agonist. For this purpose, the labeling medium was removed and the cells were rinsed once with 0.5 ml/well of wash medium (DME-H21 containing no sodium bicarbonate and supplemented with 20 mM HEPES, 40 μg/ml bacitracin, 0.1 mM phenylmethylsulfonyl fluoride, and 1 mM 3-isobutyl-1-methylxanthine). Then, 0.5 ml/well of wash medium containing the indicated agonist was added and the cells were incubated at 37° for 30 min. The medium was removed and 1 ml of ice-cold solution containing 5% trichloroacetic acid, 1 mM ATP, and 1 mM cAMP was added. The culture plates were incubated at 4° for 30 min and the soluble radioactivity was separated into ATP and cAMP fractions using Dowex cation exchange chromatography (32). The cAMP fractions were further purified by aluminum oxide chromatography as described (32). After scintillation counting of the respective fractions, the relative levels of cAMP were calculated by determining the ratio of cAMP counts to total counts (ATP plus cAMP). In this paper cAMP levels are expressed as percentage of maximal stimulation, which corresponds to the level of cAMP accumulated upon dopamine

[3] P. D. Garcia and R. M. Myers, unpublished observations.

treatment (for transfected HEK 293 cells) or vasoactive intestinal peptide treatment (for GH_3 cells).

Results

Recently, the cloning of cDNAs encoding several subtypes of SSTRs was reported (22–26). We used this sequence information to detect and clone the cDNAs of any SSTR subtypes expressed in the GH_3 cell line, a well established *in vitro* model for SSTR function. We used a PCR approach that allowed us to detect all of the known SSTR subtypes in a cDNA library from this cell line (see Experimental Procedures for details). We determined that GH_3 cells express two SSTR subtypes, which correspond to the previously identified SSTR1 and SSTR2 (22–24). From 480,000 cDNA clones screened, we found multiple cDNA isolates (58 and 48 isolates for SSTR1 and SSTR2, respectively) for each of these SSTR subtypes (data not shown). No other SSTR subtype was detected in this screening (data not shown), indicating that SSTR1 and SSTR2 are the major SSTR subtypes expressed in GH_3 cells.

The expression of both SSTR1 and SSTR2 in GH_3 cells was confirmed by Northern blot analysis (Fig. 1, *lanes 2*). We also investigated whether both SSTR1 and SSTR2 are expressed in the anterior pituitary (Fig. 1, *lanes 1*) and in the rat insulinoma cell line RINm5F (Fig. 1, *lanes 3*), which also expresses SSTRs (34). An SSTR1-specific probe revealed one major band of about 4.4 kb that was present in all three mRNA samples (Fig. 1A). This mRNA appears to be 5–10-fold more abundant in GH_3 cells than in pituitary or RINm5F cells. The size and expression in the anterior pituitary of this mRNA are in close agreement with previously reported data (24). To our knowledge, however, expression of SSTR1 in GH_3 and RINm5F cells has not been reported previously. An SSTR2-specific probe revealed two major bands of about 3.2 kb and 2.5 kb (Fig. 1B), in close agreement with previous reports (23, 35). These mRNA species are present in both pituitary and GH_3 cells, as reported previously (23, 35), but they are absent from RINm5F cells (Fig. 1B). Finally, an SSTR3-specific probe (obtained by PCR from mouse genomic DNA) failed to detect this SSTR subtype in the GH_3 cells (data not shown), in agreement with our cDNA library screening (see Experimental Procedures) and previous reports (26). SSTR3 was detected at low levels in the anterior pituitary (data not shown), as reported previously (26). These results demonstrate the presence of mRNA for both SSTR1 and SSTR2 in both the anterior pituitary and the GH_3 cell line, where the SSTRs may be involved in the regulation of secretion of growth hormone and other pituitary hormones.

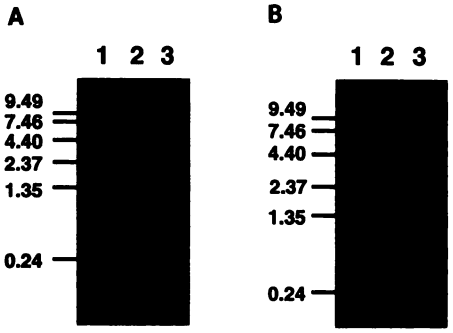

Fig. 1. Northern blot analysis of SSTR expression in the anterior pituitary. Poly(A)$^+$ RNA (2.5 μg/lane) from rat pituitary (*lanes 1*), GH_3 cells (*lanes 2*), and RINm5F cells (*lanes 3*) was fractionated by electrophoresis in 1% agarose-formaldehyde gels. After transfer of the RNA to Hybond-N$^+$ nylon membranes, the blots were hybridized with a SSTR1-specific probe (A) or with a SSTR2-specific probe (B). After high stringency washes, the blots were exposed to Kodak X-Omat film for approximately 30 hr for the exposures shown. Seven-day exposures of these blots failed to detect any signal with the SSTR2 probes from RINm5F cells (data not shown).

Somatostatin exerts its effects on GH_3 cells by inhibiting adenylyl cyclase (12) and by modifying the plasma membrane conductance for potassium (17, 18) and calcium (15). Here we tested whether either of the SSTRs expressed in GH_3 cells is capable of inhibiting adenylyl cyclase in response to somatostatin. For this purpose, we transfected expression vectors containing both SSTR cDNAs (isolated from the GH_3 cDNA library; see Experimental Procedures) into HEK 293 cells. This cell line appeared not to express functional SSTRs, because somatostatin had no effect on cAMP accumulation (Figs. 2 and 3; see Discussion for important details). HEK 293 cells were chosen because it has been previously shown that other G_i-coupled receptors inhibit cAMP accumulation when transfected into these cells (33). We transiently transfected HEK 293 cells with combinations of expression vectors encoding several G protein-coupled receptors, including SSTRs, dopamine D1 receptors, and adenosine A1 receptors. Co-transfection of the dopamine D1 receptor, which couples to stimulation of adenylyl cyclase through G_s proteins (30), allowed us to stimulate cAMP accumulation only in those cells that contained the exogenously added DNA. This is important because in transient transfections of this kind only a fraction of the cells receive DNA (see Discussion for details). The adenosine A1 receptor, which couples to inhibition of adenylyl cyclase through G_i proteins (36), was included in all of the transfections as a positive control for receptors that inhibit cAMP accumulation. Transfections with these two receptors were performed in the absence or presence of either SSTR1 or SSTR2. We then measured the cAMP accumulation in response to a variety of hormones 60–64 hr after transfection. For this purpose we labeled the cells with [^{3}H]adenine before the hormone treatments and then resolved the acid-soluble ^{3}H into ATP and cAMP fractions (see Experimental Procedures for details).

Cells incubated in the absence of hormones accumulated very small amounts of cAMP (Fig. 2). An approximately 20-fold increase in the level of cAMP was observed when the cells were incubated in the presence of 10 μM dopamine (Fig. 2). This effect was due to the transfected dopamine D1 receptor, because untransfected HEK 293 cells did not show this response (data not shown). All results were standardized to this response and expressed as a percentage of the maximal response. When transfected cells were incubated in the presence of 10 μM dopamine and 10 μM concentrations of the adenosine analog (*R*)-PIA (a specific agonist of the adenosine A1 receptor) (37), the observed accumulation of cAMP was inhibited and reached only about 40% of the maximal response (Fig. 2). This inhibitory effect of (*R*)-PIA was due to the transfected adenosine A1 receptor, because it was not observed in HEK 293 cells transfected only with the dopamine receptor (data not shown). These control responses were verified in all of our experiments. The accumulation of cAMP was also inhibited in cells transfected with SSTR1 or SSTR2 (Fig. 2) when they were treated with 10 μM dopamine and 1 μM somatostatin. This inhibitory effect of somatostatin was not observed in cells transfected with only the dopamine and adenosine receptor cDNAs (Fig. 2). These

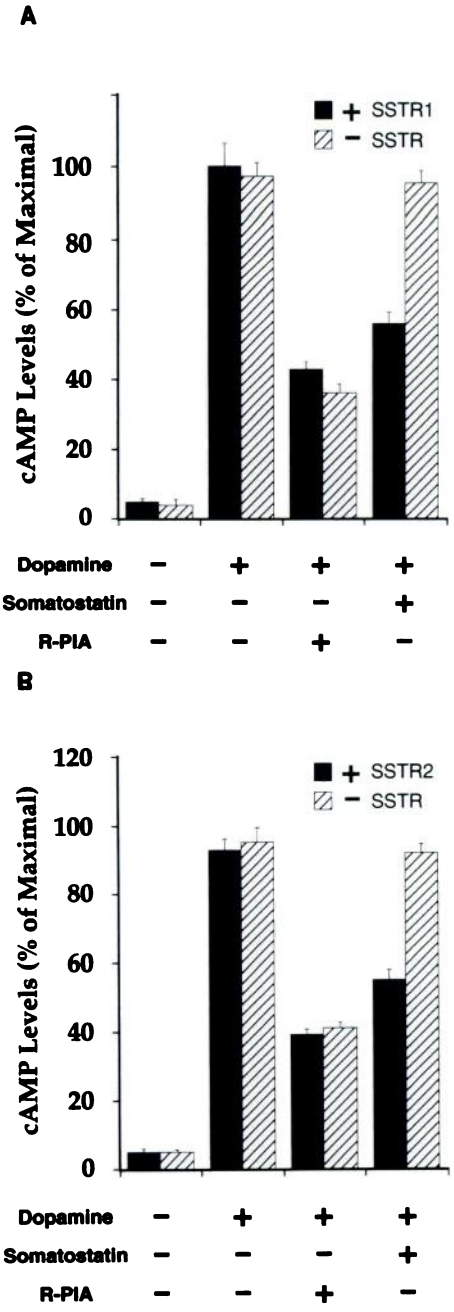

Fig. 2. Functional expression of SSTRs in HEK 293 cells. To test for the ability of SSTR1 (A) and SSTR2 (B) to inhibit cAMP accumulation, HEK 293 cells were transfected with expression vectors containing the respective cDNA. In addition to the SSTR clones, expression vectors containing the human dopamine D1 and rat adenosine A1 receptor cDNAs were co-transfected (■). Control HEK 293 cells were transfected only with cDNAs for the human dopamine D1 and rat adenosine A1 receptors (▨). The transfected cells were not treated (−) or treated (+) with the indicated hormones. The hormone concentrations used were 10 μM dopamine, 10 μM (*R*)-PIA, and 1 μM somatostatin. The extents of the responses are indicated as percentages of the maximal accumulation of cAMP when cells were treated with dopamine. The experiments shown in A and B were performed simultaneously and the maximal response refers to the SSTR1-transfected cells treated only with dopamine. Each *data point* is the average of three independent assays performed with transfected cells from the same batch, and the *error bars* represent the standard deviations. The results shown are representative of three experiments performed with different batches of transfected HEK 293 cells.

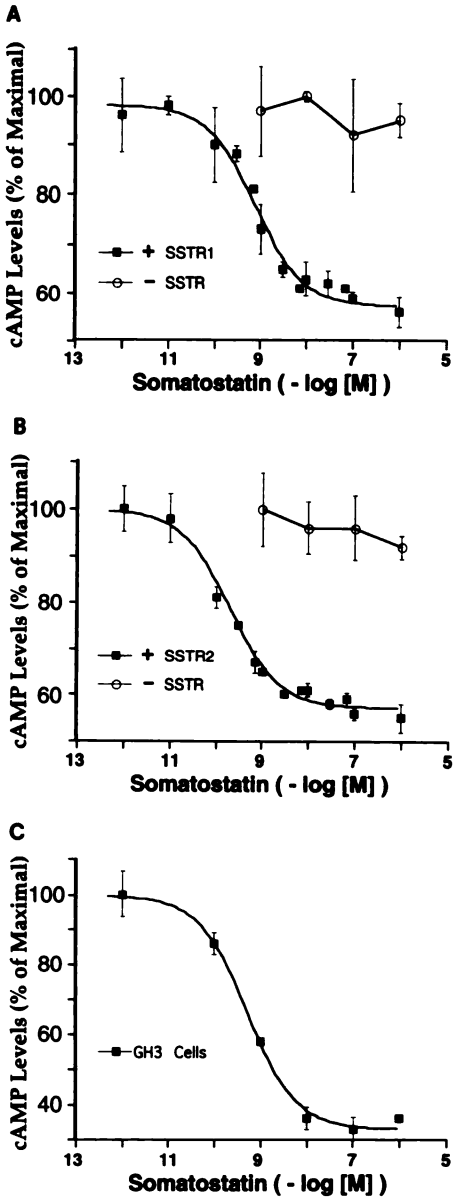

Fig. 3. Dose-response curves for somatostatin in SSTR-transfected HEK 293 and in GH_3 cells. SSTR1 (A, ■), SSTR2 (B, ■), and control (A and B, ○) transfected cells were used to measure cAMP accumulation after incubation with 10 μM dopamine and serial dilutions of somatostatin. Accumulation of cAMP in GH_3 cells (C) was measured after incubation with 0.1 μM vasoactive intestinal peptide and serial dilutions of somatostatin. Each *data point* is the average of three independent assays performed with transfected cells from the same batch and the *error bars* represent the standard deviations. The results shown in A and B are representative of two independent experiments performed with different batches of transfected HEK 293 cells. In these experiments, we obtained IC_{50} and Hill coefficients similar to those described in Results. The results in C are representative of many experiments performed with GH_3 cells, and the data are similar to the previously reported values (12). The extent of inhibition of cAMP in GH_3 cells was somewhat variable in different experiments, with cAMP accumulations ranging around 40–60% of the maximal response at the highest somatostatin concentrations. However, neither the IC_{50} nor the Hill coefficient showed significant variability.

experiments demonstrate that both SSTR1 and SSTR2 can be functionally expressed in HEK 293 cells and that both receptor subtypes inhibit adenylyl cyclase.

To further test the specificity of the somatostatin response, we carried out a dose-response experiment for both SSTR1

(Fig. 3A) and SSTR2 (Fig. 3B) in transfected HEK 293 cells. Analysis of the results showed that they fit well to the Hill equation for a noncooperative hormone/receptor interaction. The dose-response curves showed Hill coefficients of -0.8 ± 0.18 (with an IC_{50} of 0.8 nM) and -0.8 ± 0.14 (with an IC_{50} of 0.2 nM) for SSTR1 and SSTR2, respectively. As expected, no such dose-response effect was observed in HEK 293 cells transfected only with the dopamine and adenosine receptors (Fig. 3, A and B). For comparison, we performed a similar dose-response experiment with intact GH_3 cells (Fig. 3C). The results indicate a Hill coefficient of -0.8 ± 0.16 with an IC_{50} of 0.5 nM, which is close to the value reported previously (12). Thus, these results indicate that HEK 293 cells transfected with either SSTR1 or SSTR2 display approximately the same properties as those observed in intact GH_3 cells, with respect to somatostatin-induced inhibition of adenylyl cyclase.

We next tested whether the observed somatostatin inhibitory effect in HEK 293 cells transfected with SSTR1 or SSTR2 was mediated by a PTX-sensitive G protein. For this purpose SSTR1-transfected (Fig. 4A) and SSTR2-transfected (Fig. 4B) HEK 293 cells were incubated in the absence or presence of PTX, before hormone responses were assayed. Both untreated and PTX-treated cells were incubated with no hormones, with dopamine alone, with dopamine plus (*R*)-PIA, or with dopamine plus somatostatin. As expected, PTX treatment did not affect the response to dopamine alone, because it is mediated by G_s, which is insensitive to the toxin. Also as expected, the inhibitory effects of both somatostatin and (*R*)-PIA were observed in cells not treated with PTX. However, the inhibitory effects of both somatostatin and (*R*)-PIA were completely abolished in PTX-treated cells. This was true for cells transfected with SSTR1 (Fig. 4A) or SSTR2 (Fig. 4B). These results demonstrate that the inhibitory effects of adenosine A1 receptors, SSTR1, and SSTR2 in transfected HEK 293 cells are mediated by one or more PTX-sensitive G proteins, presumably of the G_i family.

The results of Figs. 2, 3, and 4 indicate that the cDNAs for SSTR1 and SSTR2 cloned from the GH_3 cell line are capable of inhibiting cAMP accumulation in transfected HEK 293 cells. The similarities of the somatostatin responses in transfected cells and intact GH_3 cells suggest that SSTR1 and/or SSTR2 are the receptor subtypes that mediate adenylyl cyclase inhibition in these cells. We directly tested this hypothesis for SSTR2, by using the specific somatostatin agonist MK-678 (38). In binding studies with SSTR-transfected cells, this analog appears to interact specifically and with high affinity (IC_{50} = 0.07 nM) only with the SSTR2 subtype (25, 39). A dose-response experiment in intact GH_3 cells showed that MK-678 effectively inhibited the accumulation of cAMP (Fig. 5). Analysis of the results indicated a Hill coefficient of -0.8 ± 0.18, with an IC_{50} of 0.1 nM. These values are in good agreement with those published for SSTR2, as determined by binding displacement experiments (25, 39). Thus, this result provides compelling evidence that SSTR2 inhibits adenylyl cyclase in GH_3 cells. Therefore, we conclude that SSTR2 (and perhaps also SSTR1) is the SSTR subtype that regulates adenylyl cyclase in the GH_3 cell line.

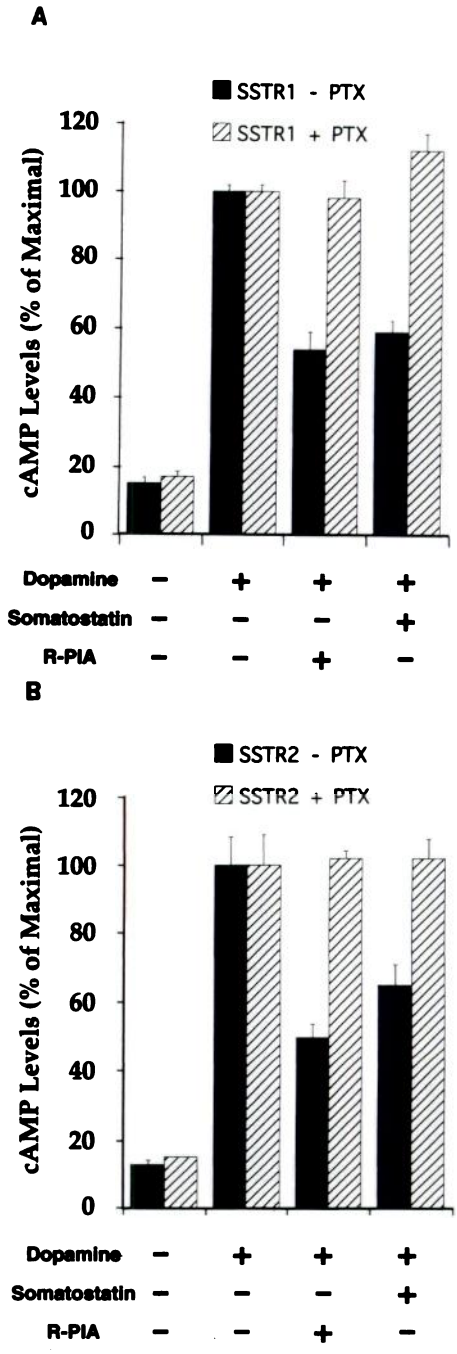

Fig. 4. SSTR1 (A) and SSTR2 (B) coupling through PTX-sensitive G_i proteins. HEK 293 cells were transfected with SSTR1 or SSTR2 as described for Fig. 2. After transfection and transfer into 24-well plates (see Experimental Procedures), half of the wells were left untreated (■), while the other half were incubated with medium containing 200 ng/ml PTX (▨). The toxin treatment was carried out for 16–20 hr, before cAMP accumulation was assayed. Hormone concentrations were the same as those used in Fig. 2. In these experiments, the relatively higher level of cAMP accumulated in the absence of hormones was due to a lower transfection efficiency (as judged by the percentage of β-galactosidase-stainable cells; see Experimental Procedures), relative to the experiment in Fig. 2. Under these conditions, the net maximal response (contributed only by transfected cells) was lower, relative to the basal levels of cAMP found in the absence of hormones (contributed by all cells). The results shown are representative of two experiments performed with different batches of transfected cells.

Discussion

We have identified and cloned cDNAs for the two major SSTR subtypes expressed in the rat pituitary cell line GH_3,

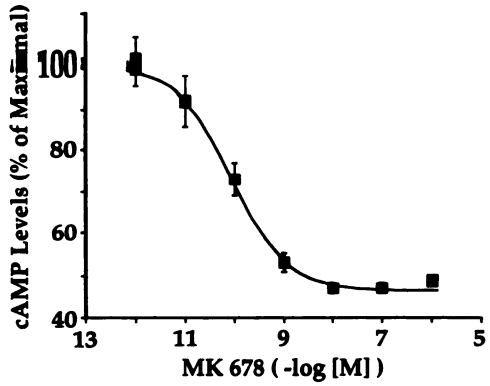

Fig. 5. Dose-response curve for MK-678 in GH_3 cells. This experiment was carried out the same way as that shown in Fig. 3C, with the exception that MK-678 (a generous gift of Dr. D. Viber, Merck Sharp & Dohme Research Laboratories) was used instead of somatostatin.

which correspond to the SSTR1 and SSTR2 subtypes (22–24). In transfection experiments, we have functionally expressed both cDNAs in HEK 293 cells and found that both receptor subtypes couple to inhibition of adenylyl cyclase through PTX-sensitive G proteins. These results indicate that functional mRNAs encoding both SSTR1 and SSTR2 are present in the GH_3 cell line, and it is likely that both play a role in the actions of somatostatin in this cell line. Our results contradict previous reports in which no inhibition of adenylyl cyclase by these SSTR subtypes was observed in COS cells (25) or CHO cells (39). These contradictory results could be explained by the different cell lines and/or the methodology used in these studies, as discussed below. Although conflicting conclusions about the ability of SSTR2 to inhibit adenylyl cyclase exist in the literature (see below), to our knowledge this is the first report of coupling of SSTR1 to inhibition of adenylyl cyclase through a PTX-sensitive G protein.

In previous work, it was demonstrated that COS cells [the cell line used by Yasuda *et al.* (25)] failed to show inhibition of cAMP accumulation when transfected with receptors that normally couple to G_i proteins, such as the α_2-adrenergic (33) and adenosine A1[4] receptors. The inhibitory effects of these receptors were observed, however, when the same vectors were transfected into HEK 293 cells (see Ref. 33 for the α_2-adrenergic receptor and Figs. 2 and 4 for the adenosine A1 receptor). We also did not observe inhibition of cAMP accumulation in SSTR1- and SSTR2-transfected COS cells (data not shown). Thus, it appears that COS cells are not suitable for this kind of experiment. One explanation for this observation is that COS cells express an adenylyl cyclase subtype that is stimulated by free $\beta\gamma$ subunits released when G_i proteins are activated (33, 40). This activation by free $\beta\gamma$ subunits has been observed with type II adenylyl cyclase when activated G_s α subunit (GTP-bound form) is also present (33, 40). This possibility was tested in HEK 293 cells by co-transfecting the α_2-adrenergic receptor with type II adenylyl cyclase (33). The results of these experiments showed that, indeed, adenylyl cyclase type II-transfected HEK 293 behave like COS cells and show no inhibition of cAMP accumulation by G_i-coupled receptors (33). Intriguingly, Yasuda *et al.* (25) observed inhibition of cAMP accumulation in COS cells transfected with the SSTR3 subtype. This observation may indicate some important difference in the coupling of SSTR3 to the cAMP second messenger pathway. For example, it is possible that SSTR3 releases different $\beta\gamma$ subunits that are not capable of stimulating the adenylyl cyclase expressed in COS cells. Therefore, further analysis of this observation may provide new insights into the complexities of these intracellular signaling pathways.

The failure to detect inhibition of cAMP accumulation in stably transfected CHO cells expressing SSTR1 and SSTR2 (10) is somewhat more difficult to explain. It is possible that loss of coupling to inhibition of adenylyl cyclase occurred during the clonal selection required to obtain stably transfected cells. If this was the case, then this problem should not be reproducible and one may expect to observe coupling in other isolates of transfected CHO cells. Indeed, this was the case with a stably transfected CHO cell line expressing SSTR2, in which somatostatin inhibited cAMP accumulation (41). Furthermore, in transient transfection experiments similar to those shown in Fig. 2 in which we used CHO cells, we observed inhibition of cAMP accumulation mediated by adenosine A1 receptor, SSTR1, and SSTR2 (data not shown). Thus, unlike in COS cells, inhibition of cAMP accumulation can occur in CHO cells transfected with SSTR1 and SSTR2. However, it appears that the SSTR-expressing CHO transfectants of Rens-Domiano and Reisine (10) have lost this property, probably during the clonal selection required for their isolation.

Recently, experiments using transfected HEK 293 cells transiently expressing SSTR2 showed a low percentage (9%) of inhibition of cAMP accumulation (29). This effect was significantly lower than those we (Figs. 2B and 3B) and Strnad *et al.* (41) have observed (40–50% and 75%, respectively). This small effect of somatostatin in transfected HEK 293 cells led those authors to conclude that SSTR2 does not inhibit adenylyl cyclase (29). However, this difference is probably due to a smaller number of cells expressing the transfected cDNAs in transient experiments. In their experiments, Law *et al.* (29) used forskolin to stimulate cAMP accumulation; somatostatin inhibited cAMP accumulation only in the fraction of cells in which DNA was introduced, whereas forskolin stimulated cAMP accumulation in all cells. Thus, these kinds of transient transfection experiments are very sensitive to the efficiency at which DNA is introduced into cells (see Experimental Procedures for details). In our transient experiment, we stimulated cAMP accumulation only in cells in which DNA was introduced by co-transfection of the dopamine D1 receptor and, therefore, we observed higher levels (40–50%) of inhibition of cAMP accumulation. Despite these differences, the results reported by those authors confirm our observation that SSTR2 does indeed couple to inhibition of adenylyl cyclase when transfected in HEK 293 cells. Interestingly, those authors demonstrated that HEK 293 cells express SSTR2 endogenously (29). However, we consistently have not observed a specific effect of somatostatin in nontransfected HEK 293 cells, indicating that the endogenous SSTR2 does not couple to inhibition of adenylyl cyclase. We have seen some small variability (see results in Fig. 3, for example) in nontransfected HEK 293 cells when somatostatin is added. However, this variability never involves a change in the levels of cAMP accumulation larger than 10% (compared with 40% inhibition in SSTR1- and SSTR2-transfected HEK 293 cells). Furthermore, this variability does not appear to correlate with the dose of somatostatin (see Fig. 3). Thus, the endogenous SSTR2 of HEK 293 cells appears to be

[4] P. D. Garcia and R. M. Myers, unpublished observations.

nonfunctional in coupling to inhibition of adenylyl cyclase, for reasons that are not understood. The fact that coupling is observed when functional SSTR2 cDNAs are transfected into HEK 293 cells indicates that all other components required for coupling are functional. This suggests that the reasons for the lack of coupling are intrinsic to the endogenous SSTR2 (perhaps a somatic mutation or an splice variant yet to be characterized).

Our PCR-based search for the SSTR subtypes expressed in GH_3 cells detected the presence of only SSTR1 and SSTR2, and their expression was confirmed by Northern blot analysis. No other SSTR subtype was detected in the PCR-based search. Furthermore, the absence of expression of the SSTR3 subtype was also confirmed by Northern blot analysis. During the later stages of our work three different research groups reported the molecular cloning of novel SSTRs (27, 28, 42), which have all been designated SSTR4. The question arises of whether any of these receptor subtypes are also expressed in GH_3 cells and, if so, whether they are the main mediators of somatostatin regulation of adenylyl cyclase. Two of these clones appear to be the rat (27) and human (28) versions of the same receptor subtype. Sequence analysis of this receptor subtype indicates that it should have been detected in the PCR-based search of the cDNA library (see Experimental Procedures). Therefore, it is likely that this receptor subtype is not represented abundantly in the GH_3 cDNA library. This negative result is consistent with the observation that this receptor subtype appears to be expressed in brain and not in pituitary or other peripheral tissues (27, 28). Moreover, this receptor subtype shows no affinity for the somatostatin analog MK-678 (27, 28) and, therefore, cannot mediate the inhibitory effect shown in Fig. 5. The other SSTR subtype recently identified was cloned from a rat pituitary cDNA library and appears to be expressed exclusively in the pituitary (42). It is not clear whether this receptor subtype could be detected in our PCR screening of the GH_3 cDNA library, because it contains some amino acid differences in one of the regions from which the oligonucleotide primers were designed. Thus, at present we do not know whether this novel SSTR subtype is expressed in GH_3 cells. However, the available pharmacological evidence indicates that this is not the receptor subtype mediating the somatostatin response observed in GH_3 cells. This novel SSTR shows differential affinities for the two naturally occurring forms of somatostatin. It has a higher affinity for the 28-amino acid form of somatostatin (somatostatin-28) than for the 14-amino acid form (somatostatin-14). CHO cells transfected with this receptor show an IC_{50} of 0.14 nM for the inhibition of cAMP accumulation for somatostatin-28, whereas they show an IC_{50} of 9.14 nM for somatostatin-14 (the hormone used in our experiments). Thus, the IC_{50} of this receptor for somatostatin-14 is at least 1 order of magnitude higher than that observed in GH_3 cells (12) (Fig. 3C). Furthermore, in binding experiments this novel pituitary-specific SSTR subtype shows an IC_{50} for MK-678 of 7.31 nM, which differs significantly from what we observed in GH_3 cells for inhibition of cAMP accumulation (Fig. 5). Taken together, all of these results strongly support our conclusion that the somatostatin-induced inhibition of cAMP accumulation in GH_3 cells is mediated through SSTR1 and/or SSTR2. Recently, the existence of two alternatively spliced forms of SSTR2 was demonstrated (43). Both forms are capable of inhibiting adenylyl cyclase but show distinct pharmacological properties (44). Our PCR-based search would not have distinguished between the alternatively spliced forms of SSTR2, because the oligonucleotide primers used in the assay are based on sequences that are common to both forms. Therefore, at present we do not know whether both forms are expressed in the GH_3 cell line. Sequence analysis of a cDNA isolated from the GH_3 cDNA library (which was used in all functional studies reported here) indicates that this clone corresponds to the SSTR2A form. We are currently testing for the presence of the SSTR2B form in GH_3 cells.

An intriguing result of the experiments described here is that a single cell line expresses two different SSTR subtypes. The biological significance of this observation is unknown at present. Northern blot analysis demonstrates that SSTR1, SSTR2, SSTR3, and the novel receptor subtype mentioned above are expressed in the anterior pituitary, suggesting that all four may have regulatory roles in pituitary. Whether more than one of these receptor subtypes is expressed in the same cell type remains to be determined. However, it is also possible that the observed pattern of SSTRs expressed in GH_3 cells is the result of the events that gave rise to the pituitary tumor from which the cells were derived or the result of propagation *in vitro*. Thus, the finding that both SSTR1 and SSTR2 are expressed in GH_3 cells may not be physiologically relevant. A rigorous answer to these questions will require the development of reagents that can detect and distinguish two or more SSTRs in a single cell *in situ*. If indeed more than one receptor is expressed in single cells *in vivo*, then a provocative hypothesis is that they may preferentially couple to different effectors or second messenger pathways.

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