0026-895X/92/06939-08$03.00/0

MOLECULAR PHARMACOLOGY, 42:939-946

ACCELERATED COMMUNICATION

Molecular Cloning and Expression of a Pituitary Somatostatin Receptor with Preferential Affinity for Somatostatin-28

ANNE-MARIE O'CARROLL, STEPHEN J. LOLAIT, MONIKA KÖNIG, and LAWRENCE C. MAHAN

Laboratory of Cell Biology, National Institute of Mental Health, Bethesda, Maryland 20892

Received August 6, 1992; Accepted September 17, 1992

SUMMARY

Using the polymerase chain reaction technique with degenerative primers, we obtained from a rat pituitary cDNA library a cDNA fragment, rAP236, that exhibited considerable homology to known receptors that belong to the guanine nucleotide-binding protein (G protein)-coupled receptor superfamily. Oligonucleotides to this fragment were used as probes to obtain a full-length cDNA from the rat pituitary cDNA library. This clone, rAP6–26, encoded a 383-amino acid protein with seven putative transmembrane domains that are characteristic of G protein-coupled receptors. The predicted amino acid sequence of the rAP6–26 cDNA exhibits 56–66% homology to recently cloned somatostatin (SRIF) receptors. Membranes prepared from COS-7 cells transfected with the rAP6–26 cDNA showed specific binding of ^{125}I-Tyr^{11}-SRIF, thus identifying the cDNA clone as a novel SRIF receptor. Radioligand binding competition analysis using somatostatin-28 (SRIF-28) and a number of cyclic SRIF analogs revealed that SRIF-28 was the most potent competitor of ^{125}I-Tyr^{11}-SRIF binding, with a ~30-fold greater affinity for the receptor than that of SRIF. In addition, binding of ^{125}I-Tyr^{11}-SRIF was markedly reduced in the presence of Na^+ ions and GTP, indicating coupling of rAP6–26 receptors to inhibitory G proteins in COS-7 membranes. In adenylyl cyclase assays, forskolin-induced cAMP accumulation was inhibited by SRIF and SRIF-28, thus confirming that the rAP6–26 cDNA encodes a functional receptor protein. By Northern blot analysis, a ~2.6 kilobase mRNA encoding the receptor was present in the pituitary but not in the liver, small intestine, kidney, pancreas, cerebellum, or cortex. Lack of receptor mRNA expression in the brain was confirmed by *in situ* hybridization histochemical studies. Thus, we report the cloning of a novel rat pituitary SRIF receptor, termed SSTR4, that has marked preferential affinity for SRIF-28 and is linked to inhibition of adenylyl cyclase.

SRIF, a tetradecapeptide widely distributed throughout the central nervous system and peripheral tissues, has been shown to act on target cells in such diverse organs as brain, pituitary, kidney, adrenals, endocrine and exocrine pancreas, and intestine (for review, see Ref. 1). The roles of SRIF as both neurotransmitter (2, 3) and hormone (1) are well documented, but perhaps the most well established neuroendocrine action of SRIF is its inhibition of growth hormone, prolactin, and thyroid-stimulating hormone secretion in the anterior pituitary (4). These actions of SRIF are mediated by high affinity, membrane-bound receptors that couple to G proteins. Functional coupling of SRIF receptors in pituitary, pancreas, and cultured cell lines (e.g., AtT-20, GH_3, and GH_4C_1 pituitary tumor cells) to adenylyl cyclase (via G_i) results in the inhibition of intracellular cAMP accumulation. Additional, pertussis toxin-sensitive, transduction pathways have been described for SRIF receptors, including activation of a hyperpolarizing K^+ conductance (G_{i3}/G_K) and inhibition of Ca^{2+} influx (G_o), similar to those described for a number of inhibitory G protein-linked receptors, e.g., muscarinic M_2, α_2-adrenergic, dopaminergic, and opiate receptors (5–9).

The existence of multiple subtypes of SRIF receptors has been suggested by the selective distribution, physiological effects, and pharmacological potency of SRIF and its biologically active, alternatively processed, precursor polypeptide, SRIF-28. SRIF-28 is a more potent inhibitor of growth hormone, prolactin, and thyroid-stimulating hormone secretion in pituitary and of pancreatic exocrine function, whereas SRIF is a more potent inhibitor of cortical neuron activity and gastric exocrine secretion (10). Further pharmacological evidence suggesting SRIF receptor multiplicity is based upon the pharmacological dis-

S.J.L. was supported by grants from the National Alliance for Research on Schizophrenia and Depression and the Stanley Foundation; A-M.O'C. was supported by a grant from Ciba Geigy.

ABBREVIATIONS: SRIF, somatotropin release-inhibiting factor/somatostatin-14; SRIF-28, somatostatin-28; PCR, polymerase chain reaction; m/hSSTR1, cloned mouse/human somatostatin 1 receptor; m/h/rSSTR2, cloned mouse/human/rat somatostatin 2 receptor; SSTR4, cloned somatostatin receptor from rat pituitary; G protein, guanine nucleotide-binding regulatory protein; G_i, inhibitory guanine nucleotide-binding protein; SSC, standard saline citrate; BSA, bovine serum albumin; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Ro20–1724, 4-(3-butoxy-4-methoxybenzyl)-2-imidazolidinone; SMS 201–995, cyclo[Phe-Cys-Phe-D-Trp-Cys-Thr-Cys-Thr-(ol)]; MK 678, cyclo[*N*-Me-Ala-Tyr-D-Trp-Lys-Val-Phe]; CGP 23996, des-Ala^1-Gly^2-[desamino-Cys^3,Tyr^{11}]-3,14-dicarbasomatostatin; CHO, Chinese hamster ovary; TM, transmembrane.

crimination of abbreviated cyclic SRIF analogs such as SMS 201–995, MK 678, and CGP 23996 for ^{125}I-Tyr^{11}-SRIF binding in brain (11). Recently, the molecular cloning of distinct cDNAs encoding SRIF receptors (termed m/hSSTR1 and m/hSSTR2) from both mouse and human genomic libraries has been reported (12). An additional report describing the rat homolog of m/hSSTR2 (which we have termed rSSTR2)[1] has appeared (13). Initial pharmacological characterization of SSTR1 and SSTR2 expressed in CHO-DG44 cells (13, 14) has demonstrated pharmacological specificity for CGP 23996 and MK 678, respectively, but no discrimination between SRIF and SRIF-28 was observed. In this communication, we describe the cloning and expression of a fourth molecular subtype of SRIF receptor (termed SSTR4)[1] from a rat pituitary cDNA library, with pharmacological properties and tissue distribution distinct from those of the molecular subtypes reported previously.

Materials and Methods

Isolation of cDNA clone. A rat pituitary cDNA library, containing ~2.8×10^6 recombinants, was constructed from poly(A)$^+$-selected RNA in the pcD2 mammalian expression vector (15, 16). Plasmid DNA (1 μg) was used as a template in a PCR reaction with 1 μM each of two degenerative primers to the TM II and VI regions of a number of G protein-coupled receptors and with *Taq* polymerase (Geneamp; Perkin Elmer-Cetus). Primer 1 was a 27-mer sense oligonucleotide with 256-fold degeneracy to the TM II region of the following receptors: rat dopamine D_2, rat α_{2b}-adrenergic, rat substance P, and rat cannabinoid receptors. Primer 2 was a 29-mer antisense oligonucleotide with 128-fold degeneracy to the TM VI region essentially as described previously (17). Forty cycles of 96° for 45 sec (denaturation), 55° for 4 min (annealing) and 72° for 4 min (extension) were carried out, followed by a final extension at 72° for 15 min. Products were analyzed on a 3% NuSieve genetic technology grade-agarose gel (FMC BioProducts). Three bands (between 544 and 610 base pairs in size) were obtained; these were excised and blunted with T4 polymerase, and terminal phosphates were added with T4 polynucleotide kinase (New England Biolabs). cDNA fragments subcloned into the *Hinc*II site of M13mp18 were sequenced and a number were found to exhibit homology with members of the G protein-coupled receptor superfamily. Sequence information from one insert, termed rAP236, was utilized to obtain a full length cDNA clone.

cDNA library screening. Two 48-base pair oligonucleotides (5′-GGAGCCTACGCGCATGCCTGCAGCCTTCACCTTGACCACAAT-GAGCAG-3′ and 5′-CAGCAGGGGCCCAAAGAAGCCCAACACA-GACTGGTAGGTGATGAAGGC-3′) were synthesized from sequences specific for the putative third intracellular loop of the PCR clone rAP236. ^{32}P-labeled oligonucleotide probes were prepared using [γ-^{32}P] ATP (3000 Ci/mmol; NEN) and T4 polynucleotide kinase and were used to screen the rat pituitary cDNA library. Pools of recombinants were screened (hybridization in 3× 20x SSC (3 M NaCl, 0.3 M sodium citrate, pH 7.0) at 60°; washing in 1× SSC at 60°) by Southern blot analysis (18). One positive cDNA clone, rAP6–26, was isolated, and overlapping fragments were subcloned into M13 and sequenced by the Sanger dideoxynucleotide chain termination method using Sequenase (United States Biochemical). Plasmid preparations were prepared and the sequence was verified by sequencing both strands on denatured double-stranded plasmid templates. Primers were derived from previously determined sequence information. Sequence analysis and comparisons were performed with genetics computer group software (University of Wisconsin) and GenBank.

[1] We have termed the rat SRIF receptor described in Reference 13, rSSTR, based on the 98% amino acid homology between this receptor and the mouse SSTR2 (12). While this manuscript was n press a third molecular subtype was described (Yasuda *et al. J. Biol. Chem.* **267**:20422–20428 (1992)), we have thus termed our somatostatin subtype SSTR4.

Cell transfection. Plasmid DNA (10–25 μg) was used to transfect COS-7 cells (ATCC CRL 1651) and CHO-K1 cells (ATCC CCL 61) by the $CaPO_4$ precipitation technique (16). Using COS-7 cells transfected with the rAP6–26 plasmid DNA, membranes were prepared for radioligand binding assays as described below. For CHO-K1 cells, selection with the neomycin analog G-418 (450 μg/ml) was started 72 hr after transfection and was continued for 3 weeks. A monoclonal line expressing the rAP6–26 cDNA was obtained by limiting dilution, and expression of rAP6–26 mRNA was determined by dot blot hybridization of cellular RNA, as described below for Northern blot analysis.

Membrane preparation and radioligand binding assays. Seventy-two hours after transfection of COS-7 cells with the rAP6–26 plasmid DNA, the cells were scraped from the culture plates in 50 mM Tris·HCl, pH 7.4, 5 mM $MgCl_2$ buffer (binding buffer), homogenized, and centrifuged (39,000 × *g*, 30 min, 4°). The crude membrane pellet was washed in the same buffer, resuspended to approximately 2–3 mg/ml, and stored frozen at −80°. Protein concentrations were determined using the Bradford protein assay (19).

Frozen membrane preparations were diluted in ice-cold binding buffer, washed once by centrifugation (39,000 × *g*, 30 min, 4°), homogenized briefly (30 sec), and resuspended to a protein concentration of approximately 0.1 mg/ml. Unless otherwise indicated, radioligand binding assays were performed in the presence of 0.1 nM ^{125}I-Tyr^{11}-SRIF (1900–2000 Ci/mmol; Amersham Corp.), appropriate unlabeled ligands, and approximately 20 μg of membrane protein, in a final volume of 250 μl of binding buffer containing 1 mg/ml BSA and 50 μg/ml bacitracin. Nonspecific binding was determined in the presence of 1 μM SRIF. All assays contained triplicate determinations. Incubations were carried out for 90 min (equilibrium) at 23° and then terminated by rapid filtration, under vacuum, through Whatman GF/B filters that had been pretreated with 0.5% polyethyleneimine/0.1% BSA. Filters were washed with 3 × 4 ml of ice-cold binding buffer and retained radioactivity was counted in a γ counter with 85% counting efficiency. SRIF, SRIF-28, arginine vasopressin, adrenocorticotropic hormone, corticotropin-releasing factor, [D-Ala^2,D-Leu^5]-enkephalin, substance P, and vasoactive intestinal peptide were purchased from Peninsula Laboratories (Belmont, CA). SMS 201–995 and MK 678 were obtained from Sandoz Pharmaceuticals Corp. (East Hanover, NJ) and Merck Sharp & Dohme Research Laboratories (Rahway, NJ), respectively. CGP 23996 was a gift of Dr. T. Reisine, University of Pennsylvania (Philadelphia, PA). Binding studies utilized COS-7 membranes prepared from four to six separate transfection groups. Data were analyzed by nonlinear least squares regression analysis using the program INPLOT 4.03 (GraphPAD, San Diego, CA). All values reported with error bars are means ± standard errors from the number of experimental determinations indicated.

Measurement of cAMP accumulation in whole cells. cAMP accumulation was measured in two ways. The first method was used to assess receptor coupling to the inhibition of adenylyl cyclase. Mouse L cells, LVIP2.0Zc, containing a cAMP-responsive β-galactosidase reporter construct (20) were transfected with putative receptor cDNAs. Agonists that bind to transiently expressed G_i-coupled receptors were identified by inhibition of forskolin-induced expression of β-galactosidase, as detected by staining of cells with a chromogenic substrate. Cells were exposed to medium containing the phosphodiesterase inhibitor Ro20–1724 (20 μM) (BioMol, Plymouth Meeting, PA) and 50 μg/ml bacitracin with forskolin alone or in combination with as many as 20 individual candidate ligands. Color development was measured at 405 nm in a 96-well plate reader. A second method was used to measure cAMP accumulation in intact cells, both COS-7 cells (transient expression) and CHO-K1 cells (stable expression), that had been transfected with clone rAP6–26, as described above. COS-7 cells were trypzined 24 hr after transfection and seeded in 12-well plates at a density of 2×10^5 cells/well. Forty-eight hours later, the growth medium was replaced with 500 μl of serum-free medium containing 20 mM HEPES, 20 μM

Ro20–1724, 1 mg/ml BSA, and 50 μg/ml bacitracin and cells were preincubated for 5 min. Either forskolin (1 μM) or forskolin plus SRIF (1 μM) was then added and the reaction was stopped after 5 min with 500 μl of an ice-cold solution containing 0.1 N HCl and 1 mM $CaCl_2$. The accumulation of cAMP was measured by radioimmunoassay as described by Brooker *et al.* (21). CHO-K1 cells were seeded directly into 12-well plates at a density of 2×10^5 cells/well and, after 24 hr, were treated exactly as described above for COS-7 cells. Mock-transfected COS-7 cells and untransfected CHO-K1 cells treated exactly as the transfected cells were used as negative controls, whereas AtT-20 cells grown to ~80% confluency were used as a positive control.

Northern blot analysis. Total RNA was extracted from rat tissues using guanidium thiocyanate and cesium trifluoroacetate (15), and poly(A)$^+$ RNA was prepared by two passes over oligo(dT)-cellulose columns. Samples (2 μg) were electrophoresed through denaturing 2.2 M formaldehyde/1% agarose gels and transfered to a S&S Nytran membrane (Schleicher & Schuell). The blots were UV-cross-linked and prehybridized at 37° for 24 hr in 4× standard saline phosphate EDTA (3.6 M NaCl, 0.2 M sodium phosphate, pH 7.7, 20 mM EDTA), 50% formamide, 5× Denhardts' solution (0.1% borine serum albumin, 0.1% Ficoll 400, 0.1% polyvinylpyrrolidone), 500 μg/ml single-stranded salmon sperm DNA, 250 μg/ml yeast tRNA, 0.1% sodium dodecyl sulfate. Hybridization was carried out (in the aforementioned buffer) for 18 hr at 37° with the same 48-base pair oligodeoxynucleotide probes described above, which had been tailed on the 3′ end with terminal deoxynucleotidyl transferase (Boehringer Mannheim) and [α-^{32}P] dATP (3000 Ci/mmol; NEN). The blot was washed for 4 × 15 min at 60° in 1x SSC/0.1% sodium dodecyl sulfate and then exposed to Kodak XAR film for 5 days at −80° with intensifying screens.

Results and Discussion

In an ongoing attempt to clone novel G protein-coupled receptors present in the pituitary, PCR methodology was used with degenerative primers to amplify potential receptor clones. One PCR fragment, rAP236, was pursued because it exhibited considerable homology to known G protein-coupled receptors. Using sequence-specific probes, a full length cDNA termed rAP6–26 was isolated from a rat pituitary cDNA library. The clone rAP6–26 contains an open reading frame that encodes a 383-amino acid polypeptide with a calculated relative molecular mass of ~48,000. Fig. 1 shows the partial nucleotide sequence (1149 base pairs) and deduced amino acid sequence of the cDNA.[2] There are a number of possible sites for post-translational modification of this cloned receptor. One potential *N*-linked glycosylation site (Asn-X-Ser/Thr, where X is any amino acid) is present at Asn-13 in the putative extracellular amino terminal and a second is at Asn-186 in the second extracellular loop. The carboxyl terminus contains a number of serine/threonine (six/two) residues that could serve as a substrate for serine/threonine protein kinases. The initation codon and the surrounding nucleotides agree well with the consensus initation sequence (22). Hydrophobicity analysis of the translated protein reveals seven putative hydrophobic TM domains that are characteristic of G protein-coupled receptors. The predicted third intracellular loop, thought to be involved in coupling to G proteins, is only 28 amino acids in length. Sequence comparison analysis with cloned receptors of the G protein-coupled superfamily revealed that, in the TM domains, rAP6–26 has ~56% sequence identity with the recently reported mSSTR1 (12) and ~66% identity with the rSSTR2 (13). The regions of highest homology are TM2, TM3, and TM7, whereas those of least homology are the putative extracellular loops and the amino and carboxyl termini. rAP6–26 exhibits a 64% and 36% sequence divergence in the third intracellular loop, compared with mSSTR1 and rSSTR2, respectively (Fig. 2).

The marked amino acid homology between clone rAP6–26 and both mSSTR1 and rSSTR2, as well as preliminary functional screening data obtained in LVIP2.0Zc cells (see below), suggested that the putative receptor protein encoded by the rAP6–26 cDNA belonged to the SRIF receptor family. The rAP6–26 cDNA was, therefore, transiently expressed in COS-7 cells in order to examine the ligand-binding specificity of the rAP6–26 protein. Membranes prepared from these cells were initially examined for binding in the presence of 0.1 nM levels of the SRIF agonist ^{125}I-Tyr^{11}-SRIF and were compared with binding in membranes prepared from rat forebrain and mock-transfected COS-7 cells (Fig. 3A). Membranes from rat forebrain and rAP6–26-transfected COS-7 cells bound ^{125}I-Tyr^{11}-SRIF to a similar magnitude and degree (%) of specific binding (168.5 ± 8.5 fmol/mg, 78.5 ± 2.9%, three experiments, and 181 ± 40.7 fmol/mg, 73.4 ± 3.7%, six experiments, respectively). In contrast, binding of ^{125}I-Tyr^{11}-SRIF to mock-transfected or untransfected COS-7 membranes was very low (36.9 ± 4.0 fmol/mg, three experiments), with a high degree of nonspecific binding (52.4 ± 6.7%, three experiments). In subsequent binding experiments it was determined that the low, apparently specific, binding of ^{125}I-Tyr^{11}-SRIF to mock-transfected or untransfected COS-7 membranes, determined in the presence of 1 μM SRIF, was unrelated to the binding properties (e.g., pharmacological specificity) observed in either rat forebrain or rAP6–26-transfected COS-7 cells (data not shown). Thus, introduction of rAP6–26 cDNA into COS-7 cells produced an almost 6-fold increase in the specific binding of ^{125}I-Tyr^{11}-SRIF. Similar results were observed by transfection and transient expression of rAP6–26 cDNA in human embryonic kidney 293 cells carrying SV40 T antigen and COS-1 cells (ATCC CRL 1650) (data not shown).

Further identification and characterization of the putative SRIF receptor expressed in COS-7 cells was carried out by radioligand binding competition analysis using SRIF-28 and a number of cyclic SRIF analogs (Fig. 3B). Analysis of the IC_{50} values of the competitive displacement curves revealed that SRIF-28 was the most potent competitor of ^{125}I-Tyr^{11}-SRIF binding, displaying an approximate 30-fold greater affinity than SRIF (Fig. 3B; Table 1). In addition, high affinity displacement was observed for the cyclic SRIF analog SMS 201–995 (23) and the subtype-selective cyclic analogs CGP 23996 (24) and MK 678 (25). This pharmacological profile contrasts with those obtained for recently reported SRIF receptor clones expressed in the central nervous system and peripheral tissues (12–14). In all cases Hill coefficients derived from the competitive agonist displacement data were less than unity (ranging from 0.43 to 0.57), suggestive of multiple agonist affinity states as have been reported previously for receptors in membrane preparations that couple to G proteins. Curves could be best described by high (low picomolar, 50–80%) and low (low nanomolar, 20–50%) affinity components, although interpretation of these data is complex in view of the predicted ability of ^{125}I-Tyr^{11}-SRIF to bind with some fractional occupancy to each of these affinity states at the concentration used (see below). In contrast, a number of unrelated peptides, adrenocorticotropic hormone, arginine vasopressin, corticotropin-releasing factor,

[2] The nucleotide sequence reported in this paper has been deposited in the GenBank databank (accession number L04535).

Fig. 1. Nucleotide sequence for the rAP6–26 cDNA and deduced amino acid sequence for the rat SSTR4. The nucleotide sequence is numbered at the *right*. The deduced amino acid sequence is shown beneath the nucleotide sequence; amino acid numbering begins with the first methionine of the long open reading frame. An in-frame stop codon in the 5′ untranslated region is *underlined*. Positions of the putative TM segments I-VII are indicated by *solid lines above the nucleotide sequence*; the termini of each segment are tentatively assigned on the basis of the hydrophobicity profile and sequence comparison with other G protein-coupled receptors.

[D-Ala^2,D-Leu^5]-enkephalin, substance P, and vasoactive intestinal peptide, that similarly interact at G protein-coupled receptors were ineffective at displacing ^{125}I-Tyr^{11}-SRIF bound to COS-7 membranes transfected with rAP6–26 cDNA. Taken together, the radioligand binding data in Fig. 3 and Table 1 indicate that rAP6–26 cDNA encodes a rat pituitary SRIF receptor with marked preferential affinity for SRIF-28 and little or no discrimination between CGP 23996 and MK678. Another PCR clone, with ~92% nucleotide identity (over the 270 base pairs sequenced) with the rAP6–26 cDNA, has been obtained from a cDNA library derived from AtT-20 cells,[3] which also display preferential affinity for SRIF-28 in functional and radioligand binding assays.

SRIF receptors, like a number of G protein-coupled receptors linked to the inhibition of adenylyl cyclase, display diminished affinity for agonists specifically in the presence of either Na^+ ions or GTP (26–28). The site of allosteric modulation by Na^+ has been shown by site-directed mutagenesis in α_2-adrenergic receptors to reside in the highly conserved aspartate residue in TM II (28), which is also present in position 83 in rAP6–26. Additional experiments were, therefore, performed to determine whether the binding of ^{125}I-Tyr^{11}-SRIF to rAP6–26-transfected COS-7 membranes was sensitive to the presence of GTP and Na^+ ions (Fig. 4A). Inclusion of either 100 μM GTP or 100 mM Na^+ reduced the equilibrium binding of ^{125}I-Tyr^{11}-SRIF by ~55%. Inclusion of both GTP and Na^+ markedly reduced binding by ~83%, indicating that the SRIF receptor encoded by rAP6–26 likely couples to G proteins (G_i or G_o) in COS-7 membranes. Identical results have been observed for the effects of Na^+ and GTP on the binding of ^{125}I-CGP 23996 to SRIF receptors on AtT-20 pituitary tumor cells (29) and to solubilized SRIF receptors (30). The reduction in equilibrium binding observed in the presence of GTP and/or Na^+ was attributable to an acceleration of the dissociation of ^{125}I-Tyr^{11}-SRIF bound to receptor (Fig. 4B). In addition, the observed biphasic dissociation of ^{125}I-Tyr^{11}-SRIF from control rAP6–26 membranes supports the notion of multiple agonist affinity states suggested by the Hill coefficients obtained for the various somatostatin agonists in competitive radioligand binding experiments (Fig. 3B). These results may explain the extremely low binding of ^{125}I-Tyr^{11}-SRIF observed in intact CHO-DG44 cells transfected with hSSTR1 and mSSTR2 cDNAs, which was measured in the presence of 150 mM NaCl at 22° (12). Therefore, based upon sequence homologies and the unique pharmacological

[3] S. J. Lolait, unpublished observations.

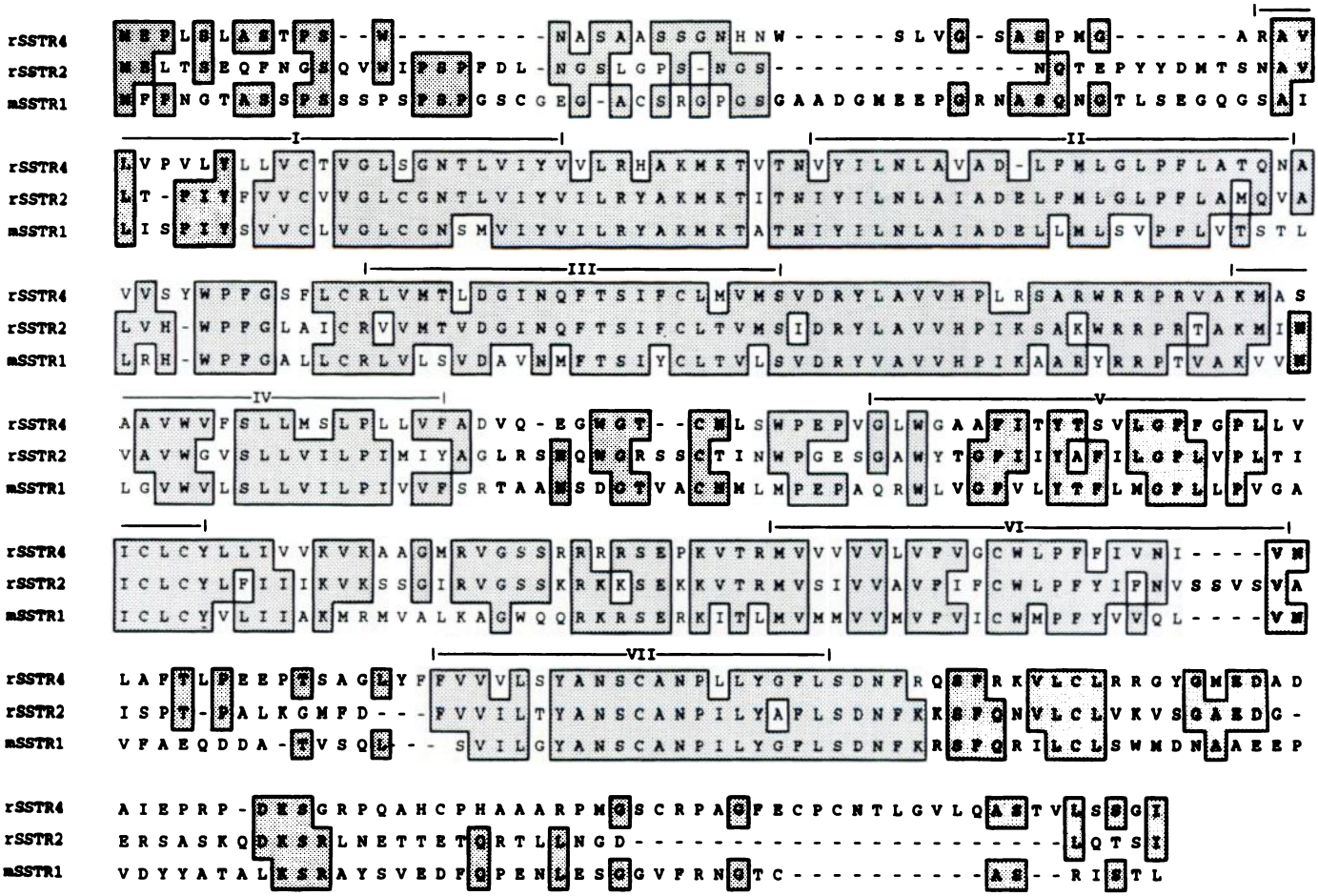


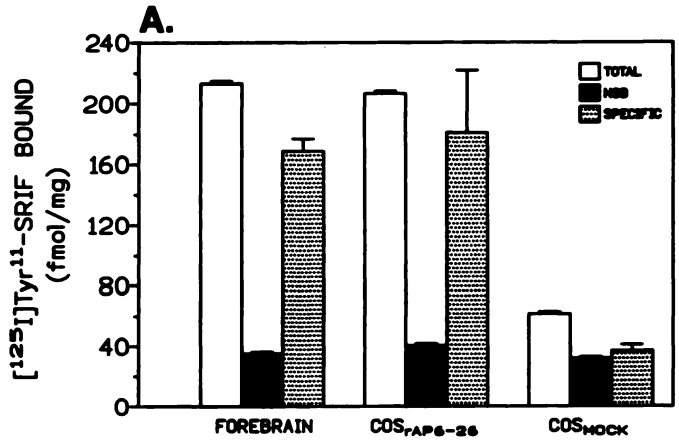
Fig. 2. Comparison of the deduced amino acid sequence of the rat SSTR4 with the previously characterized mouse SSTR1 and rat SSTR2. *Dashes*, gaps introduced to maximally align sequences. Primary sequence identities are *boxed and shaded*. The proposed seven membrane-spanning hydrophobic sequences (labeled *I–VII*) are indicated by *solid lines*.

properties of the SRIF receptor encoded by rAP6–26, we have termed this fourth molecular SRIF subtype SSTR4.

During initial attempts to identify candidate ligands for clone rAP6–26, it was observed that SRIF (1 μM), specifically out of a dozen ligands screened, attenuated forskolin (0.5 μM)-stimulated β-galactosidase expression in LVIP2.0Zc cells (data not shown). Preliminary studies in COS-7 cells that transiently expressed SSTR4 indicated that both SRIF and SRIF-28 decreased forskolin (1 μM)-stimulated cAMP accumulation by 20–23%, which is consistent with values obtained in brain (31) and pituitary cells in culture (32). In CHO-K1 cells stably transfected with SSTR4, both SRIF and SRIF-28 inhibited forskolin (2 μM)-stimulated cAMP accumulation in a dose-dependent manner (Fig. 5). SRIF-28 (IC_{50}, 0.144 nM) was a more potent inhibitor of forskolin-stimulated accumulation of cAMP than was SRIF (IC_{50}, 9.14 nM) which agrees well with the radioligand data obtained in this study. It is also possible that the binding of SRIF to SSTR4 may activate other transduction mechanisms, as is likely to be the case for hSSTR1 and mSSTR2, which do not appear to couple to the inhibition of adenylyl cyclase when expressed in CHO-DG44 cells (14).

To characterize SSTR4 further, the tissue distribution of its corresponding mRNA was examined by Northern blot analysis in rat tissues. One labeled band, corresponding to a mRNA of ~2.6 kilobases, was found only in the pituitary (Fig. 6). No detectable RNA species were observed in the liver, small intestine, kidney, pancreas, cortex, or cerebellum. *In situ* hybridization with two ^{35}S-labeled 48-base pair oligodeoxynucleotide probes (data not shown) confirmed the presence of mRNA expression in the pituitary, with no detectable hybridization found in the brain, kidney, heart, adrenal, or spleen. Thus, the distribution of SSTR4 mRNA contrasts with that of hSSTR1 (12) and rSSTR2 (13); the former is present in jejunum, stomach, pancreatic islets, kidney, and colon and the latter is found in the cortex, hippocampus, and pancreatic tumor cells in addition to pituitary. It should be noted, however, that species-specific differences in SRIF receptor mRNA distribution may exist because, by Northern blot analysis, human but not rat kidney appears to express mRNA encoding SSTR2 (12, 13).

Two lines of pharmacological and functional evidence have established the existence of multiple subtypes of SRIF receptors. Initially, separate subtypes with preferential affinity for either SRIF or SRIF-28 were proposed and were supported by regional differences in the binding of ^{125}I-SRIF and ^{125}I-SRIF-28, in physiological potency, and in the ability of SRIF and SRIF-28 to selectively down-regulate receptors in different tissues and cultured cell lines (for review, see Ref. 10). In this regard, the pituitary and pituitary-derived cell lines, such as AtT-20, demonstrate preferential affinity (3–10-fold) for SRIF-28 in functional and radioligand binding assays. The apparent



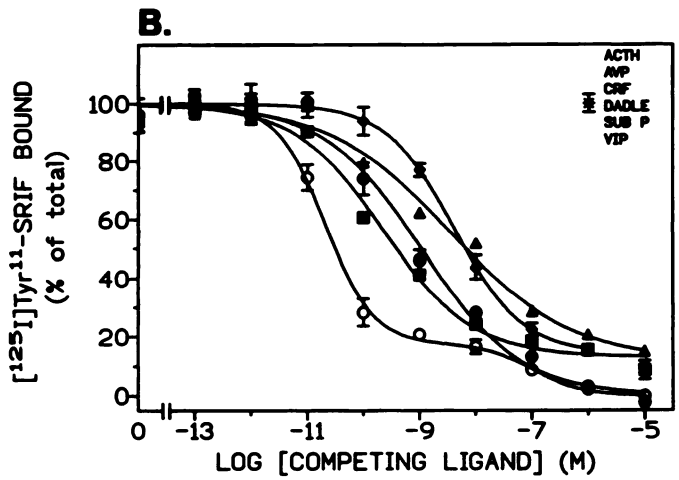


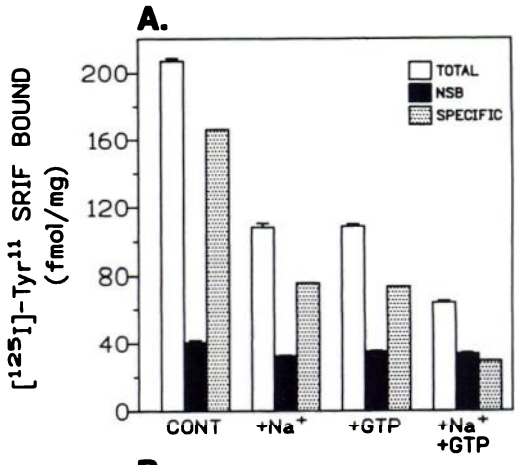
Fig. 3. Binding of ^{125}I-Tyr^{11}-SRIF to membranes prepared from COS-7 cells transfected with rAP6–26 cDNA. A, Total, nonspecific (*NSB*), and specific binding of ^{125}I-Tyr^{11}-SRIF to membranes prepared from rat forebrain, rAP6–26-transfected COS-7 cells (*COS-7$_{rAP6-26}$*), and mock-transfected COS-7 cells (*COS-7$_{MOCK}$*). Membranes were incubated with 0.1 nM ^{125}I-Tyr^{11}-SRIF (75,000–85,000 cpm), and nonspecific binding was determined in the presence of 1 μM SRIF. Total and nonspecific data shown are from a single representative experiment; specific binding data were derived from three to six separate experiments and values are reported in Results. B, Competitive radioligand binding assays on membranes prepared from rAP6–26-transfected COS-7 cells. Membranes were incubated with 0.1 nM ^{125}I-Tyr^{11}-SRIF and the indicated concentrations of SRIF (●), SRIF-28 (○), SMS 201–995 (■), MK 678 (▲), or CGP 23996 (♦). A number of other peptides (*) did not show any displacement of ^{125}I-Tyr^{11}-SRIF binding (also see Results). Data shown are from a single representative experiment; values from three experiments are given in Table 1.

TABLE 1

Comparison of inhibition by SRIF, SRIF-28, and cyclo-somatostatin analogs of binding of ^{125}I-Tyr^{11}-SRIF to rSSTR4 expressed in COS-7 cells

Values are mean ± standard error (three experiments).

Competing ligand	IC_{50}	Hill coefficient	Relative affinity
	nM		
SRIF	2.58 ± 0.91	0.44 ± 0.05	1.0
SRIF-28	0.087 ± 0.048	0.49 ± 0.12	0.034
SMS 201-995	0.187 ± 0.36	0.45 ± 0.07	0.072
MK 678	7.31 ± 2.12	0.43 ± 0.02	2.83
CGP 23996	4.74 ± 1.49	0.57 ± 0.08	1.84



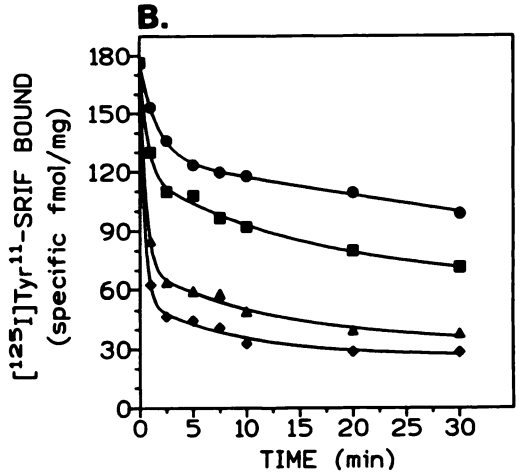


Fig. 4. Effects of GTP and Na^+ ions on the binding of ^{125}I-Tyr^{11}-SRIF to membranes prepared from rAP6–26-transfected COS-7 cells. A, Equilibrium binding assays were performed in the presence of 100 μM GTP, 100 mM NaCl, or both. Membranes were incubated with 0.1 nM ^{125}I-Tyr^{11}-SRIF and nonspecific binding (*NSB*) was determined in the presence of 1 μM SRIF. B, Dissociation of ^{125}I-Tyr^{11}-SRIF bound to membranes in the absence (●) or presence of 100 μM GTP (▲), 100 mM NaCl (■), or both (♦). Membranes were incubated with 0.05 nM ^{125}I-Tyr^{11}-SRIF (nonspecific binding was determined in the presence of 1 μM SRIF) for 90 min at 23°. Aliquots (250 μl) (in triplicate) from either total or nonspecific batch incubations were then diluted into 2 ml of binding buffer containing either GTP, NaCl, or both and 0.1 μM SRIF and were incubated further at 23°. Dissociation was terminated by filtration at the times indicated. Data shown are representative of two experiments performed on COS-7 membranes prepared from separate rAP6–26 transfections.

pituitary-specific localization of mRNA for SSTR4 and marked preferential affinity for SRIF-28 strongly implicate it as the candidate "SRIF-28" receptor. In contrast, although moderate levels of mRNA for the rat homolog of SSTR2 (13) were demonstrated in pituitary (and we have independently cloned a partial-length SSTR2 cDNA from the rat pituitary cDNA library used in this study[4]), rSSTR2 showed no differences in affinity for SRIF and SRIF-28 in competitive radioligand binding assays.

Further evidence for the multiplicity of subtypes of SRIF

[4] S. J. Lolait, M. J. Brownstein, and A.-M. O'Carroll, unpublished observations.

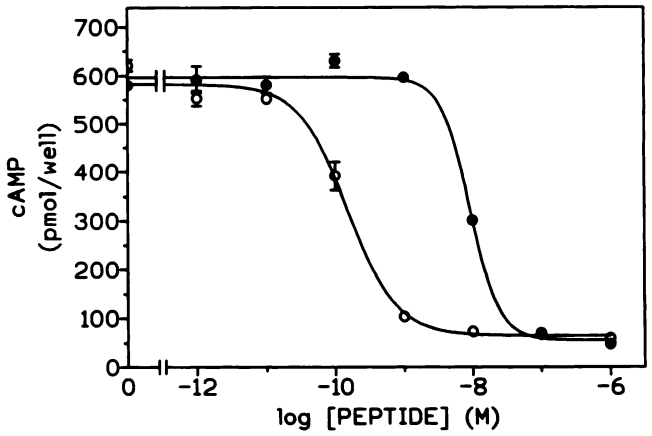


Fig. 5. SRIF- and SRIF-28-induced inhibition of forskolin-stimulated cAMP accumulation in rAP6–26-transfected CHO-K1 cells. CHO-K1 cells were seeded in 12-well plates at a density of 2×10^5, and after 24 hr the inhibition of forskolin (2 μM)-stimulated cAMP accumulation produced by the indicated concentrations of SRIF (●) and SRIF-28 (○) was measured as described in Materials and Methods. Data shown are the mean ± standard error compiled from two separate experiments, with each stimulation/inhibition performed in triplicate. (Basal cAMP levels = 61.16 ± 3.30 pmol/well (n = 12)). SRIF and SRIF-28 did not inhibit cAMP accumulation in nontransfected cells (data not shown).

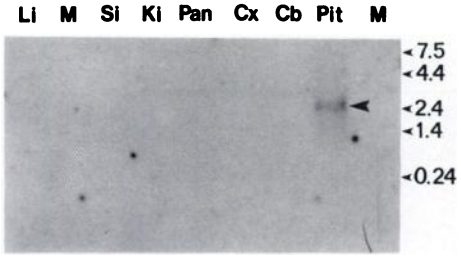


Fig. 6. Northern blot analysis of the rat SSTR4 mRNA in rat tissues. Poly(A)$^+$ RNA samples (2 μg) were analyzed on a denaturing 2.2 M formaldehyde/1% agarose gel as described in Materials and Methods. *Large arrowhead*, ~2.6 kilobase receptor mRNA. RNA molecular markers in kilobases (BRL) are indicated on the *right*. Only the pituitary (*Pit*) expresses the receptor mRNA after a 5-day exposure. Other tissues shown are liver (*Li*), small intestine (*Si*), kidney (*Ki*), pancreas (*Pan*), cortex (*Cx*), and cerebellum (*Cb*). *M*, Molecular markers. The ^{32}P-labeled probes used in this analysis are complementary to sequence encoding amino acids 199–214 and 221–236 of rat SSTR4 and correspond to regions that are not highly conserved among the mouse and rat SSTR1 and SSTR2 sequences.

receptors was later proposed by Tran *et al.* (33), who demonstrated marked biphasic displacement by SMS 201–995 of the binding of ^{125}I-SRIF analogs in rat brain membranes. More convincing evidence, however, has been demonstrated by Reisine and co-workers (11, 34). Regional differences in the binding of MK 678 and CGP 23996 in brain and in potencies for SRIF receptors (termed SRIF$_1$-MK 678 sensitive and SRIF$_2$-MK 678 insensitive, respectively) have been described. Most recently, the binding properties of hSSTR1 and mSSTR2 expressed in CHO-DG44 cells were investigated (14). SSTR1 displays no affinity for MK 678 (and is, therefore, characteristic of SRIF$_2$ receptors), whereas SSTR2 binds ^{125}I-MK 678 with high affinity and the binding could be displaced equally by SRIF, SRIF-28, SMS 201–995, and CGP 23996 but not by CGP analogs compounds 1–3 (and SSTR2 is, therefore, characteristic of SRIF$_1$ receptors). In this regard, however, the pituitary, unlike the brain, appears pharmacologically homogeneous, demonstrating high affinity binding of both MK 678 and CGP 23996 (11, 35). These latter results agree well with the observed pharmacological properties of the cloned SSTR4 and its slightly higher sequence homology to SSTR2.

Taken together, molecular and pharmacological evidence underscores the existence of multiple SRIF subtypes in pituitary. The apparent lack of difference in affinity for SRIF and SRIF-28 observed for SSTR2, unlike SSTR4, and the presence of both SSTR2 and SSTR4 mRNAs encoding proteins of molecular weights of 41,000 and 48,000 (nonglycosylated), respectively, support results obtained from cross-linking and autoradiographic studies that suggest that physically distinct SRIF receptors are present in the pituitary (36, 37). Additional evidence has also been provided by Kimura *et al.* (38), who have described two physically and functionally distinct SRIF receptor subtypes in the rat anterior pituitary, one of which is exclusively expressed by pretreatment with 17β-estradiol. It seems unlikely, however, that SSTR4 represents the 17β-estradiol-dependent SRIF receptor subtype present on mammotrophs because, unlike the latter, SSTR4 displays marked differences in affinity for SRIF and SRIF-28.

For the present, the existence and combined pharmacological properties of SSTR2 and SSTR4 appear to account for data obtained on the properties of SRIF receptors in intact and dissociated pituitary by a variety of methods. Additional studies on the relative amounts and cellular distribution of these two SRIF receptors, and the regulation of expression of their cognate mRNAs in pituitary, will enhance our understanding of their roles in hypothalamic-pituitary interactions.

Acknowledgments

We thank M. J. Brownstein for oligonucleotide preparation and cDNA library construction.

References

1. Reichlin, S. Somatostatin. *N. Engl. J. Med.* **309**:1495–1501, 1556–1563 (1983).
2. Epelbaum, J. Somatostatin in the central nervous system: physiological and pathological modifications. *Prog. Neurobiol.* **27**:63–100 (1986).
3. Dichter, M. A., H. L. Wang, and T. Reisine. Electrophysiological effects of somatostatin-14 and somatostatin-28 on mammalian central nervous system neurons. *Metabolism* **39** (Suppl. 2):86–90 (1990).
4. Patel, Y. C., and C. B. Srikant. Somatostatin mediation of adenohypophysial secretion. *Annu. Rev. Physiol.* **48**:551–567 (1986).
5. Enjalbert, A. Multiple transduction mechanisms of dopamine, somatostatin and angiotensin II receptors in anterior pituitary cells. *Horm. Res.* **31**:6–12 (1989).
6. Schonbrunn, A. Somatostatin action in pituitary cells involves two independent transduction mechanisms. *Metabolism* **39**:96–100 (1990).
7. Reisine, T. Cellular mechanisms of somatostatin inhibition of calcium influx in the anterior pituitary cell line AtT-20. *J. Pharmacol. Exp. Ther.* **254**:646–651 (1990).
8. Wang, H. L., T. Reisine, and M. Dichter. Somatostatin-14 and somatostatin-28 inhibit calcium currents in rat neocortical neurons. *Neuroscience* **38**:335–342 (1990).
9. Rens-Domiano, S., and T. Reisine. Biochemical and functional properties of somatostatin receptors. *J. Neurochem.* **58**:1987–1996 (1992).
10. Patel, Y. C., K. K. Murthy, E. E. Escher, D. Banville, J. Spiess, and C. B. Srikant. Mechanism of action of somatostatin: an overview of receptor function and studies of the molecular characterization and purification of somatostatin receptor proteins. *Metabolism* **39**:63–69 (1990).
11. Raynor, K., and T. Reisine. Analogs of somatostatin selectively label distinct subtypes of somatostatin receptors in rat brain. *J. Pharmacol. Exp. Ther.* **251**:510–517 (1989).
12. Yamada, Y., S. R. Post, K. Wang, H. S. Tager, G. I. Bell, and S. Seino. Cloning and functional characterization of a family of human and mouse somatostatin receptors expressed in brain, gastrointestinal tract, and kidney. *Proc. Natl. Acad. Sci. USA* **89**:251–255 (1992).
13. Kluxen, F.-W., C. Bruns, and H. Lübbert. Expression cloning of a rat brain somatostatin receptor cDNA. *Proc. Natl. Acad. Sci. USA* **89**:4618–4622 (1992).
14. Rens-Domiano, S., S. F. Law, Y. Yamada, S. Seino, G. I. Bell, and T. Reisine. Pharmacological properties of two cloned somatostatin receptors. *Mol. Pharmacol.* **42**:28–34 (1992).
15. Okayama, H., M. Kawaichi, M. Brownstein, F. Lee, T. Yokota, and K. Arai.

High-efficiency cloning of full-length cDNA: construction and screening of cDNA expression libraries for mammalian cells. *Methods Enzymol.* **154**:3–28 (1987).
16. Chen, C., and H. Okayama. High-efficiency transformation of mammalian cells by plasmid DNA. *Mol. Cell. Biol.* **7**:2745–2752 (1987).
17. Libert, F., M. Parmentier, A. Lefort, C. Dinsart, J. Van Sande, C. Maenhaut, M. J. Simmons, J. E. Dumont, and G. Vassart. Selective amplification and cloning of four new members of the G protein-coupled receptor family. *Science (Washington D. C.)* **244**:569–572 (1989).
18. Bonner, T. I., N. J. Buckley, A. C. Young, and M. R. Brann. Identification of a family of muscarinic acetylcholine receptor genes. *Science (Washington D. C.)* **237**:527–532 (1987).
19. Bradford, M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**:248–254 (1976).
20. König, M., L. C. Mahan, J. W. Marsh, J. S. Fink, and M. J. Brownstein. Method for identifying ligands that bind to cloned G_s- or G_i-coupled receptors. *Mol. Cell. Neurosci.* **2**:331–337 (1991).
21. Brooker, G., J. F. Harper, W. L. Teraski, and R. O. Moylan. Radioimmunoassay of cyclic AMP and cyclic GMP. *Adv. Cyclic Nucleotide Res.* **10**:1–33 (1979).
22. Kozak, M. Compilation and analysis of sequences upstream from the translational start site in eukaryotic mRNAs. *Nucleic Acids Res.* **12**:857–872 (1984).
23. Bauer, W., U. Briner, W. Doepfner, R. Haller, R. Huguenin, P. Marbach, T. J. Petcher, and J. Pless. SMS 201-995: a very potent and selective octapeptide analogue of somatostatin with prolonged action. *Life Sci.* **31**:1133–1140 (1982).
24. Czernik, A., and B. Petrack. Somatostatin receptor binding in rat cerebral cortex: characterization using a non-reducible somatostatin analog. *J. Biol. Chem.* **258**:5525–5530 (1983).
25. Veber, D., R. M. Friedlinger, D. S. Perlow, W. J. Paleveda, Jr., F. W. Holly, R. G. Strachan, R. F. Nutt, B. H. Arison, C. Hommick, W. C. Randall, M. S. Glitzer, R. Saperstein, and R. Hirschmann. A potent cyclic hexapeptide analogue of somatostatin. *Nature (Lond.)* **292**:55–58 (1981).
26. Taparel, D., J. P. Esteve, C. Susini, N. Vaysse, D. Balas, G. Berthon, E. Wunsch, and A. Ribet. Binding of somatostatin to guinea-pig pancreatic membranes: regulation by ions. *Biochem. Biophys. Res. Commun.* **115**:827–833 (1983).
27. Enjalbert, A., R. Rasolonjanahary, E. Moyse, C. Kordon, and J. Epelbaum. Guanine nucleotide sensitivity of [^{125}I]-iodo NTyr somatostatin binding in rat adenohypophysis and cerebral cortex. *Endocrinology* **113**:822–824 (1983).
28. Horstman, D. A., S. Brandon, A. L. Wilson, C. A. Guyer, E. J. Cragoe, Jr., and L. E. Limbird. An aspartate conserved among G-protein receptors confers allosteric regulation of α_2-adrenergic receptors by sodium. *J. Biol. Chem.* **265**:21590–21595 (1990).
29. Mahan, L. C., and T. Reisine. Molecular mechanisms of somatostatin inhibition of hormone release from AtT-20 cells, in *Somatostatin: Basic and Clinical Status* (S. Reichlin, ed.). Plenum Press, New York, 137–145 (1987).
30. Reisine, T., H.-T. He, S. Rens-Domiano, J.-M. Martin, K. Raynor, S. Borislow, and K. Thermos. Biochemical properties of somatostatin receptors. *Metabolism* **39**:70–73 (1990).
31. Raynor, K., H.-L. Wang, M. Dichter, and T. Reisine. Subtypes of brain somatostatin receptors couple to multiple cellular effector systems. *Mol. Pharmacol.* **40**:248–253 (1991).
32. Epelbaum, J., F. Agid, Y. Agid, A. Beaudet, P. Bertrand, A. Enjalbert, V. Heidet, C. Kordon, S. Krantic, J. F. Leonard, F. Musset, E. Moyse, F. Peillon, A. Slama, and C. Videau. Somatostatin receptors in brain and pituitary. *Horm. Res.* **31**:45–50 (1989).
33. Tran, V., F. Beal, and J. Martin. Two types of somatostatin receptors differentiated by cyclic somatostatin analogs. *Science (Washington D. C.)* **228**:492–495 (1985).
34. Martin, J.-L., M.-F. Chesselet, K. Raynor, C. Gonzales, and T. Reisine. Differential distribution of somatostatin receptor subtypes in rat brain revealed by newly developed somatostatin analogs. *Neuroscience* **41**:581–593 (1991).
35. Enjalbert, A., L. Tapia-Arancibia, M. Rieutort, P. Brazeau, C. Kordon, and J. Epelbaum. Somatostatin receptors on rat anterior pituitary membranes. *Endocrinology* **110**:1634–1640 (1982).
36. Morel, G., P. Leroux, and G. Pelletier. Localization and characterization of somatostatin-14 and somatostatin-28 receptors in the rat pituitary as studied by slide-mounted frozen sections. *Neuropeptides* **6**:41–52 (1985).
37. Lewis, L. D., and J. A. Williams. Structural characterization of the somatostatin receptor in rat anterior pituitary membranes. *Endocrinology* **121**:486–492 (1987).
38. Kimura, N., C. Hayafuji, and N. Kimura. Characterization of 17-β-estradiol-dependent and -independent somatostatin receptor subtypes in rat anterior pituitary. *J. Biol. Chem.* **264**:7033–7040 (1989).

Send reprint requests to: Dr. Anne-Marie O'Carroll, Laboratory of Cell Biology, Building 36, Room 3A-17, National Institutes of Health, 9000 Rockville Pike, Bethesda, MD 20892.