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Splice Variant of the Somatostatin Receptor 2 Subtype, Somatostatin Receptor 2B, Couples to Adenylyl Cyclase

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SUMMARY

The diverse biological actions of somatostatin (SRIF) are mediated by a family of receptors, of which five have been cloned and characterized. One of the SRIF receptor subtypes, SSTR2, has been shown to exist in two forms. SSTR2A and SSTR2B are 369 and 346 amino acids in size, respectively, and differ in length and amino acid sequence in their intracellularly located carboxyl termini. SSTR2A and SSTR2B are generated by alternative splicing of SSTR2 mRNA. We previously characterized mouse SSTR2A and showed that it could be distinguished from other cloned SRIF receptor subtypes by its high affinity for MK-678 and its lack of coupling to adenylyl cyclase. To determine whether the properties of mouse SSTR2A and SSTR2B differ, we have expressed both in COS-7 cells and characterized their ligand-binding properties and ability to couple to adenylyl cyclase. The two receptors exhibited similar affinities for a number of SSTR2-selective agonists such as MK-678. Pretreatment with SRIF of COS-7 cells expressing each receptor reduced high affinity agonist binding to both SSTR2A and SSTR2B, indicating that both receptors can be regulated. Furthermore, agonist binding to both receptors was reduced by GTP analogs and Na^+, indicating that they both associate with G proteins. As shown previously, SSTR2A could not mediate SRIF inhibition of forskolin-stimulated cAMP formation. In contrast, SSTR2B was coupled to adenylyl cyclase and was able to mediate SRIF inhibition of forskolin-stimulated cAMP formation. Thus, SSTR2A and SSTR2B differ in their ability to couple to adenylyl cyclase. Because SSTR2A and SSTR2B differ only in the length and amino acid sequence of their carboxyl termini, these findings imply that the carboxyl-terminal 15 residues of SSTR2B may be involved in coupling this receptor to adenylyl cyclase.

SRIF is a cyclic tetradecapeptide that has diverse actions in the brain and endocrine system (1, 2). It was originally characterized as the major physiological inhibitor of growth hormone release from the anterior pituitary (1). The actions of SRIF are mediated by a family of receptors (3). Five SRIF receptors have recently been cloned and the different SRIF receptor subtypes have been designated SSTR1 through SSTR5 (4–13). Recent studies have suggested that SSTR2 selectively mediates the inhibition of growth hormone secretion by SRIF (14).

SRIF is believed to inhibit growth hormone release in part by blocking adenylyl cyclase activity in somatotrophs (2). In previous studies, we have shown that mouse SSTR2 couples to the pertussis toxin-sensitive G proteins $G_{i\alpha 3}$ and $G_{o\alpha 2}$ (15) but does not mediate inhibition of cAMP formation (15, 16). This finding was unexpected, because selective agonists at SSTR2, such as MK-678, inhibit cAMP formation in anterior pituitary and pituitary-derived cell lines (17–19).

Recently, a splice variant of SSTR2 was cloned from the mouse neuroblastoma × rat glioma hybrid cell line NG108 (8). This variant, SSTR2B, was generated by alternative splicing of SSTR2 mRNA and differs from the unspliced form (SSTR2A) with respect to length and sequence of the carboxyl-terminal domain. Mouse SSTR2A and SSTR2B are proteins of 369 and 346 amino acids, respectively. Their sequences are identical from residue 1 to residue 331 (Fig. 1). Both forms of SSTR2 mRNA are expressed in most tissues, although their relative abundance varies (8).

To determine whether differences exist in the properties of the variants of SSTR2, SSTR2A and SSTR2B were expressed in COS-7 cells and tested for their ligand specificities, their regulation by agonist pretreatment and guanine nucleotides, and their coupling to adenylyl cyclase. The ligand specificities of SSTR2A and SSTR2B were similar, both receptors could be regulated by agonist pretreatment, and both associated with G proteins. However, SSTR2B coupled to adenylyl cyclase in COS-7 cells and mediated SRIF inhibition of cAMP formation.

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ABBREVIATIONS: SRIF, somatostatin; PCR, polymerase chain reaction; Gpp(NH)p, guanosine-5′-(β,γ-imido)triphosphate; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid.

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                                                                    <----- M1 -----
mSSTR1 1 MFPNGTASSPSSSPSPSPGSCGEGACSRGPGSGAADGMEEPGRNASQNGTLSEGQGSAILISFIYSVVCLVG
mSSTR2A              1 MEMSSEQLNGSQVWVSSPFDLNGSLGPSNGSNQTEPYYDMTSNAVLTFIYFVVCVVG
mSSTR2B              1 MEMSSEQLNGSQVWVSSPFDLNGSLGPSNGSNQTEPYYDMTSNAVLTFIYFVVCVVG
mSSTR3             1 MATVTYPSSEPMTLDPGNTSSTWPLDTTLGNTSAGASLTGLAVSGILISLVYLVVCVVG
rSSTR4                   1 MEPLSLASTPSWNASAASSGNHNWSLVGSASPMGARAVLVPVLYLLVCTVG
rSSTR5               1 MNTPATLPLGGEDTTWTPGINASWAPDEEEDAVRSDGTGTAGMVTIQCIYALVCLVG

          ----------->          <-------- M2 -------->               <--- M3 ----
mSSTR1    LCGNSMVIYVILRYAKMKTATNIYILNLAIADELLMLSVPFLVTSTLLRH:WPFGALLCRLVLSVDAVNMFT
mSSTR2A   LCGNTLVIYVILRYAKMKTITNIYILNLAIADELFMLGLPFLAMQVALVH:WPFGKAICRVVMTVDGINQFT
mSSTR2B   LCGNTLVIYVILRYAKMKTITNIYILNLAIADELFMLGLPFLAMQVALVH:WPFGKAICRVVMTVDGINQFT
mSSTR3    LLGNSLVIYVVLRHTSSPSVTSVYILNLALADELFMLGLPFLAAQNALSY:WPFGSLMCRLVMAVDGINQFT
rSSTR4    LSGNTLVIYVVLRHAKMKTVTNVYILNLAVADVLFMLGLPFLATQNAVVSYWPFGSFLCRLVMTLDGINQFT
rSSTR5    LVGNALVIFVILRYAKMKTATNIYLLNLAVADELFMLSVPFVASAAALRH:WPFGAVLCRAVLSVDGLNMFT

          ---------->                    <-------- M4 --------->
mSSTR1    SIYCLTVLSVDRYVAVVHPIKAARYRRPTVAKVVNLGVWVLSLLVILPIVVFSRTAANSDGT:VACNMLMPE
mSSTR2A   SIFCLTVMSIDRYLAVVHPIKSAKWRRPRTAKMINVAVWCVSLLVILPIMIYAGLRSNQWGR:SSCTINWPG
mSSTR2B   SIFCLTVMSIDRYLAVVHPIKSAKWRRPRTAKMINVAVWCVSLLVILPIMIYAGLRSNQWGR:SSCTINWPG
mSSTR3    SIFCLTVMSVDRYLAVVHPTRSARWRTAPVARTVSRAVWVASAVVVLPVVVFSGVP:::RGM:STCHMQWPE
rSSTR4    SIFCLMVMSVDRYLAVVHPLRSARWRRPRVAKMASAAVWVFSLLMSLPLLVFADVQ:EGWGT:::CNLSWPE
rSSTR5    SVFCLTVLSVDRYVAVVHPLRAATYRRPSVAKLINLGVWLASLLVTLPIAVFADTRPARGGEAVACNLHWPH

               <---------- M5 ---------->                                    <--
mSSTR1    PAQRWLVGFVLYTFLMGFLLPVGAICLCYVLIIAKMRMVALKAGW::::::::::QQRKRSERKITLMVMM
mSSTR2A   ESGAWYTGFIIYAFILGFLVPLTIICLCYLFIIIKVKSSGIRVGS::::::::::SKRKKSEKKVTRMVSI
mSSTR2B   ESGAWYTGFIIYAFILGFLVPLTIICLCYLFIIIKVKSSGIRVGS::::::::::SKRKKSEKKVTRMVSI
mSSTR3    PAAAWRTAFIIYMAALGFFGPLLVICLCYLLIVVKVRSTTRRVRAPSCQWVQAPACQRRRRSERRVTRMVVA
rSSTR4    PVGLWGAAFITYTSVLGFFGPLLVICLCYLLIVVKVKAAGMRVGS::::::::::SRRRRSEPKVTRMVVV
rSSTR5    P::AWSAVFVIYTFLLGFLLPVLAIGLCYLLIVGKMRAVALRAGW::::::::::QQRRRSEKKITRLVLM

          -------- M6 -------->          <--------- M7 ---------->
mSSTR1    VVMVFVICWMPFYVVQLVNVF::AEQDDATVSQ::LSVILGYANSCANPILYGFLSDNFKRSFQRILCLSWM
mSSTR2A   VVAVFIFCWLPFYIFNVSSVSVAISPTPALKGMFDFVVILTYANSCANPILYAFLSDNFKKSFQNVLCLVKV
mSSTR2B   VVAVFIFCWLPFYIFNVSSVSVAISPTPALKGMFDFVVILTYANSCANPILYAFLSDNFKKSFQNVLCLVKA
mSSTR3    VVALFVLCWMPFYLLNIVNVVCPLPEEPAFFGLYFLVVALPYANSCANPILYGFLSYRFKQGFRRILLRPSR
rSSTR4    VVLVFVGCWLPFFIVNIVNLAFTLPEEPTSAGLYFFVVVLSYANSCANPLLYGFLSDNFRQSFRKVLCLRRG
rSSTR5    VVTVFVLCWMPFYVVQLLNLFV::TSLDATVNH::VSLILSYANSCANPILYGFLSDNFRRSFQRVLCLRCC

mSSTR1    DNAAEEPVDYYATALKSRAYSVEDFQPENLESGGVFRNGTCASRISTL - 391
mSSTR2A   SGTEDGERSDSKQDKSRLNETTETQRTLLNGDLQTSI - 369
mSSTR2B   DNSQSGAEDIIAWV - 346
mSSTR3    RIRSQEPGSGPPEKTEEEEDEEEEERREEEERRMQRGQEMNGRLSQIAQAGTSGQQPRPCTGTAKEQQLLPQ
rSSTR4    YGMEDADAIEPRPDKSGRPQATLPTRSCEANGLMQTSRI - 363
rSSTR5    LLETTGGAEEEPLDYYATALKSRGGPGCICPPLPCQQEPMQAEPACKRVPFTKTTTF - 384

mSSTR3    EATAGDKASTLSHL - 428
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Fig. 1. Comparison of amino acid sequences of the cloned SRIF receptors. The sequences of the cloned mouse (*m*) and rat (*r*) subtypes are shown. Invariant residues are shown in *boldface* type. *Colons*, gaps introduced to generate this alignment. The seven predicted transmembrane domains (*M1-M7*) are shown. The sequences are from Refs. 4–8.

TABLE 1

Affinity of SSTR2A and SSTR2B for SRIF analogs

Values are the means of three different experiments, and the standard error was <10% of the mean.

Compound	IC_{50}	
	SSTR2A	SSTR2B
	nM	
D-Trp^8-SRIF	0.001	0.001
MK-678	0.01	0.01
SMS-201-995	0.4	0.2
BIM 23023	0.001	0.001
BIM 23027	0.001	0.001
BIM 23034	0.001	0.001
NC4-28B	0.001	0.001
L362-862	0.23	0.6
L363-572	6.0	8.5

In contrast, SRIF did not inhibit cAMP formation in cells expressing SSTR2A. Because SSTR2A and SSTR2B differ in sequence in only a limited region at their carboxyl termini, this finding implicates this region of SSTR2 in coupling to adenylyl cyclase.

Experimental Procedures

Materials. SRIF and SRIF-28 were obtained from Bachem (Torrance, CA). MK-678, L-363,572, and L-362,862 were the gifts of Dr. D. Veber (Merck, West Point, PA). SMS-201-995 was obtained from Sandoz (Basel, Switzerland). All other peptides were the gifts of Dr. D. Coy (Tulane University, New Orleans, LA) and Biomeasure, Inc. (Hopkinton, MA).

Cloning of mouse SSTR2B. A SSTR2B cDNA construct was engineered by the PCR-based strategy, using SSTR2A cDNA as a template. The 3′ half of SSTR2A cDNA was first PCR amplified with oligo-$m_2$51 (nucleotides 1191–1210 of SSTR2B) and oligo-$m_2$52 (nucleotides 1557–1579 of SSTR2B). To generate a corresponding fragment for the 3′ half of SSTR2B cDNA, the PCR product was reamplified with oligo-$m_2$51 and oligo-$m_2$50 (nucleotides 1557–1629 of SSTR2B), tagged with a *Bam*HI site at the 5′ end of the primer; oligo-$m_2$50 covers the divergent region between SSTR2A and SSTR2B. The PCR was carried out for 25 cycles of denaturation at 95° for 1 min, annealing at 55° for 1 min, and extension at 72° for 1 min, using GeneAmp reagents. The amplified fragments were digested with *Kpn*I and *Bam*HI and subcloned into pGEM3Z (yielding pGEM3Z-3′2B). The *Xba*I/*Kpn*I fragment from SSTR2A and the *Kpn*I/*Sal*I fragment from pGEM3Z-3′2B were subcloned into the *Xba*I/*Sal*I site of pCMV6C to generate pCMV-SSTR2B. The sequence of this fragment was identical to the published SSTR2B cDNA sequence (8). Both cDNAs were transfected into COS-7 cells as described previously (5, 16).

Receptor binding assay. Binding studies were performed using the same procedures as described previously (14, 16). Cells were harvested 72 hr after transfection in 50 mM Tris·HCl, pH 7.8, 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) and were centrifuged at 24,000 × *g* for 7 min at 4°. The pellet was homogenized in buffer 1 using a Brinkman Polytron (setting 2.5, 30 sec). The homogenate was then centrifuged at 48,000 × *g* for 20 min at 4°. The

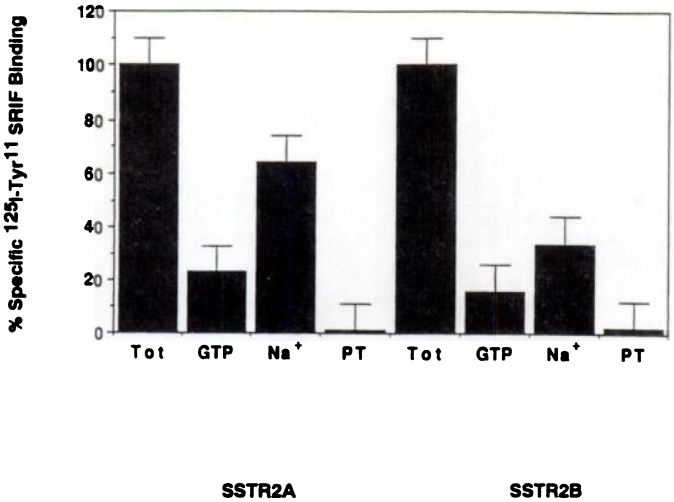

Fig. 2. Regulation of agonist binding to SSTR2A and SSTR2B by agonist pretreatment, GTP analogs, and Na^+. In membranes from COS-7 cells transfected with either SSTR2A or SSTR2B, high affinity ^{125}I-Tyr^{11}-SRIF (0.2 nM) binding was inhibited by either 100 μM Gpp(NH)p (*GTP*) or 90 mM Na^+. In other experiments, COS-7 cells expressing each receptor were pretreated for 1 hr with 100 nM SRIF (*PT*) and washed extensively, and then specific ^{125}I-Tyr^{11}-SRIF binding to the cell membranes was analyzed and compared with binding in nontreated cells. Average specific ^{125}I-Tyr^{11}-SRIF binding (*Tot*) to SSTR2A was 2500 ± 200 cpm/10 μg of protein tissue and to SSTR2B was 2000 ± 250 cpm/10 μg of protein. Values are the mean ± standard error of three different experiments.

pellet was homogenized in buffer 1 and the membranes were used in the radioligand binding assay. Cell membranes (approximately 10 μg of protein) were incubated with ^{125}I-Tyr^{11}-SRIF (0.2 nM; specific activity, 2000 Ci/mmol; NEN) in the presence or absence of competing peptides, in a final volume of 200 μl, for 30 min at 25°. Nonspecific binding was defined as the radioactivity remaining bound in the presence of 100 nM SRIF. The binding reaction was terminated by the addition of ice-cold 50 mM Tris·HCl buffer, pH 7.8, and rapid filtration with 12 ml of ice-cold Tris·HCl buffer, and the bound radioactivity was counted in a γ counter (80% efficiency). Data from radioligand binding studies were used to generate inhibition curves. IC_{50} values were obtained from curve-fitting performed with the mathematical modeling program FITCOMP (20), available through the National Institutes of Health-sponsored PROPHET system.

To determine the effects of previous exposure of the receptors to high concentrations of agonist, cells were incubated for 1 hr in the presence or absence of 1 μM SRIF. The cell culture medium was then removed, and cells were washed twice, harvested, and assayed as described above. We previously demonstrated that the loss of agonist labeling is not simply due to a masking of sites by residual peptide (18).

cAMP accumulation studies. Studies examining the potencies of these peptides to inhibit forskolin-stimulated cAMP accumulation were performed as described previously (5, 15, 16). Briefly, cells used for cAMP accumulation studies were subcultured in 12-well culture plates. COS-7 cells were transfected 72 hr before the experiments. Culture medium was removed from the wells and replaced with 500 μl of fresh medium containing 0.5 mM isobutylmethylxanthine. Cells were incubated for 20 min at 37°. Medium was then removed and replaced with fresh medium containing 0.5 mM isobutylmethylxanthine, with or without 10 μM forskolin and various concentrations of peptides. Cells were incubated for 30 min at 37°. Medium was then removed, and cells were sonicated in the wells in 500 μl of 1 N HCl and frozen for subsequent determination of cAMP content by radioimmunassay. Samples were thawed and diluted in cAMP radioimmunassay buffer before analysis of cAMP content using the commercially available assay kit from NEN/DuPont (Wilmington, DE).

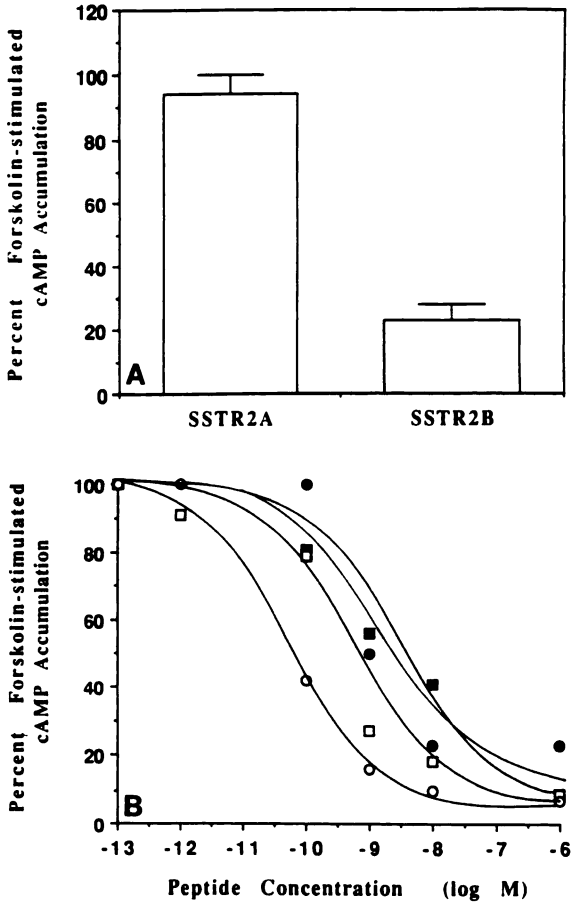

Fig. 3. Coupling of SSTR2B to adenylyl cyclase. A, COS-7 cells expressing SSTR2A or SSTR2B were stimulated for 30 min with 10 μM forskolin in the presence or absence of 1 μM SRIF. The cells were then washed, and the cAMP was extracted and analyzed by radioimmunoassay. The inhibition of forskolin-stimulated cAMP accumulation is expressed as a percentage of control. In SSTR2A-containing cells, basal cAMP levels were 5 ± 3 pmol of cAMP/well and forskolin-stimulated values were 63 ± 9 pmol of cAMP/well. In SSTR2B-expressing cells, basal cAMP was 3 ± 2 pmol of cAMP/well and forskolin-stimulated values were 45 ± 7 pmol/well. The values are the mean ± standard error of three different experiments. B, Forskolin-stimulated cAMP accumulation in COS-7 cells expressing SSTR2B was inhibited by various concentrations of SRIF (●), SRIF-28 (■), MK-678 (○), and SMS-201-995 (□). The IC_{50} values for inhibition of forskolin-stimulated cAMP accumulation were as follows: MK-678, 50 pM; SMS-201-955, 600 pM; SRIF, 2 nM; SRIF-28, 3 nM. These are the averaged values of two different experiments.

Results

Mouse SSTR2A and SSTR2B were expressed in COS-7 cells. Both receptor forms were labeled with ^{125}I-Tyr^{11}-SRIF and binding was inhibited by a series of SRIF analogs that were previously shown to interact potently with SSTR2A (14, 21). The compounds tested bound to the two forms of SSTR2 with similar potencies (Table 1).

Previous studies have shown that SRIF pretreatment of cells expressing SSTR2A reduces high affinity agonist binding to this receptor (16). Both SSTR2A and SSTR2B can be regulated by agonist pretreatment. Pretreatment of COS-7 cells expressing either SSTR2A or SSTR2B with 100 nM SRIF for 1 hr reduced high affinity ^{125}I-Tyr^{11}-SRIF binding to each receptor by >90% (Fig. 2). The procedures used to wash the treated cells ensured that the reduced binding was not due to excess peptide

TABLE 2
Properties of the six cloned SRIF receptors

	Mouse SSTR1	Mouse SSTR2A	Mouse SSTR2B	Mouse SSTR3	Rat SSTR4	Human SSTR5
GTP sensitivity	No	Yes	Yes	Yes	Yes	No
Agonist regulation	No	Yes	Yes	Yes	Yes	Yes
Adenylyl cyclase coupling	No	No	Yes	Yes	Yes	No

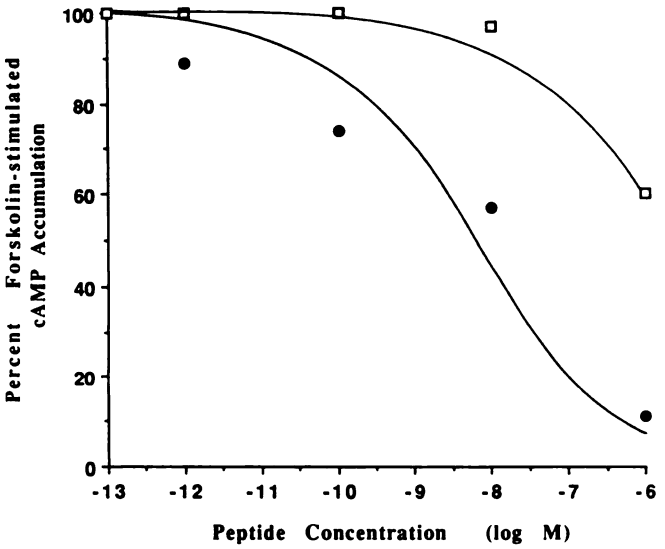

Fig. 4. Desensitization of SSTR2B. COS-7 cells expressing SSTR2B were treated for 2 hr in the presence (□) or absence (●) of 1 μM SRIF. The cells were washed and then stimulated with 10 μM forskolin in the presence or absence of SRIF. These are the averaged results of two different experiments.

associated with the receptors but instead reflected a loss of high affinity agonist binding sites in the membranes (18).

Agonist binding to both receptors was regulated by GTP analogs. Gpp(NH)p (100 μM) reduced ^{125}I-Tyr^{11}-SRIF binding to SSTR2A and SSTR2B by 80% (Fig. 2), indicating that both receptors associate with G proteins. This is further indicated by the ability of 90 mM Na^+ to reduce high affinity agonist binding to both receptors (Fig. 2) as shown previously (22).

In COS-7 cells expressing SSTR2A, 1 μM SRIF did not inhibit forskolin-stimulated cAMP accumulation (Fig. 3A), nor did SRIF-28, MK-678, or SMS-201-995 (data not shown). Furthermore, lower concentrations (0.1–100 nM) of these peptides did not inhibit forskolin-stimulated cAMP formation in COS cells expressing SSTR2A (data not shown). These findings are consistent with previous reports that SSTR2A does not couple to adenylyl cyclase (3, 5, 15, 16). In contrast, in COS-7 cells expressing SSTR2B, SRIF inhibited forskolin-stimulated cAMP accumulation by >80% (Fig. 3A). Furthermore, SRIF, SRIF-28, MK-678, and SMS-201-995 potently inhibited cAMP formation in these cells, with IC_{50} values of 5 nM or less (Fig. 3B). Pretreatment of COS-7 cells expressing SSTR2B with 1 μM SRIF greatly reduced the potency of SRIF to inhibit cAMP formation, indicating that the cAMP response to SRIF desensitizes (Fig. 4). These findings show that SSTR2B couples to adenylyl cyclase, whereas SSTR2A does not.

Discussion

The two different forms of the SRIF receptor subtype SSTR2 have similar affinities for SRIF and other SRIF analogs, and both are coupled to G proteins. However, they may be linked to different cellular effector systems. The results presented in this report indicate that SSTR2B is coupled to adenylyl cyclase via G proteins. We previously reported that SSTR2A does not couple to adenylyl cyclase (3, 5, 15, 16), and we have proposed that it may couple to ion channels via G proteins (3, 15); however, this hypothesis needs to be confirmed directly. SRIF receptors associate with multiple G proteins, including $G_{i\alpha1}$, $G_{i\alpha3}$, and $G_{o\alpha}$ (23). Of these, $G_{i\alpha1}$ specifically couples SRIF receptors to adenylyl cyclase (24). SSTR2A interacts selectively with $G_{i\alpha3}$ and $G_{o\alpha}$ (15), and the lack of association with $G_{i\alpha1}$ may explain its inability to mediate agonist inhibition of cAMP formation.

Our previous studies (5, 15, 16) reporting that the cloned mouse SSTR2A did not mediate agonist inhibition of cAMP formation were in contrast to other reports showing that SSTR2-selective agonists, including MK-678, can inhibit adenylyl cyclase activity in brain and anterior pituitary as well as in anterior pituitary-derived cells such as AtT-20 and GH_3 (17–19). We had interpreted these results to argue for the presence in these tissues of another SRIF receptor subtype that can be activated by high concentrations of SSTR2-selective agonists such as MK-678 (3). The present studies resolve this issue, because they show that SSTR2A and SSTR2B, which are coexpressed in most tissues (8), have identical ligand-binding properties but SSTR2B couples to adenylyl cyclase, whereas SSTR2A does not. Moreover, they indicate that the carboxyl-terminal 15 amino acids of SSTR2B play an important role in this interaction. Consistent with this possibility, Sugimoto *et al.* (25) recently reported that the EP_3 receptor mRNA undergoes alternative splicing to generate two receptors that differ in amino acid sequence only in their carboxyl termini. These receptors interact differently with G proteins and adenylyl cyclase, indicating that the carboxyl terminus of the EP_3 receptor, like that of SSTR2, may be a critical domain responsible for adenylyl cyclase coupling.

The amino acid sequence of the carboxyl-terminal region of SSTR2B, which couples to adenylyl cyclase, is very different from that of SSTR3 or SSTR4 (Fig. 1), other SRIF receptor subtypes that are also coupled to adenylyl cyclase (5, 6, 21). This implies that there is not a single conserved linear motif that denotes whether or not a SRIF receptor subtype will be coupled to adenylyl cyclase.

There is now evidence for six different SRIF receptors, two of which, SSTR2A and SSTR2B, are products of the same gene and are generated by alternative splicing. The properties of the different SRIF receptors are summarized in Table 2. It will be important to determine whether alternative splicing of the other receptor subtypes generates additional functional diversity.

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