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Somatostatin

Terry Reisine[1,2]

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SUMMARY

1. Somatostatin (SRIF) exerts diverse physiological actions in the body including regulation of hormone and neurotransmitter release and neuronal firing activity. Analogs of SRIF are used clinically to treat tumors and cancers and to block the hypersecretion of growth hormone in acromegaly.

2. The recent cloning of five SRIF receptor subtypes has allowed for the identification of the molecular basis of the cellular actions of SRIF. The ligand binding domains and regions involved in coupling to G proteins and cellular effector systems are being identified and the processes by which SRIF inhibits cell growth and proliferation are being established. Furthermore, subtype selective agonists have been generated which are being used to investigate the specific biological roles of each SRIF receptor subtypes.

3. Such information will be useful in developing a new generation of SRIF drugs that could be employed to treat metabolic diseases, disorders of the gut, cancer and abnormalities in the central nervous system such as epilepsy and Alzheimer's disease.

INTRODUCTION

Somatostatin (SRIF) is a 14 amino acid containing peptide that is expressed throughout the central and peripheral nervous systems as well as different organ (Brazeau *et al.*, 1972; Epelbaum, 1986; Epelbaum *et al.*, 1994; Raynor and Reisine, 1992; Reichlin, 1983). It is involved in the regulation of the release of a number of hormones and neurotransmitters. It inhibits the secretion of growth

[1] Department of Pharmacology, University of Pennsylvania School of Medicine, 36th Street and Hamilton Walk, Philadelphia, Pennsylvania 19104.
[2] To whom correspondence should be addressed.

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hormone, thyroid stimulating hormone and prolactin from the anterior pituitary and reduces the release of insulin and glucagon from islet cells and amylase secretion from acinar cells of the pancreas (Brazeau *et al.*, 1972; Epelbaum, 1986; Gomez-Pan *et al.*, 1975; Hellman and Lernmark, 1969; Reichlin, 1983). SRIF also modulates the release of catecholamines and indoleamines in the brain and in particular was reported to stimulate the release of dopamine in the striatum (Chesselet and Reisine, 1983; Epelbaum, 1986; Raynor and Reisine, 1992).

A higher molecular form of SRIF, somatostatin-28 (SRIF-28) is also biologically active. SRIF-28 consists of a 14 amino acid N-terminal extention of SRIF (Pradayrol *et al.*, 1980). Like SRIF, SRIF-28 has been shown to inhibit hormone secretion from different organs and to regulate neurotransmission (Epelbaum, 1986; Raynor and Reisine, 1992; Mandarino *et al.*, 1981; Brown *et al.*, 1977).

SRIF and SRIF-28 are derived from prosomatostatin which is a biologically inactive precursor. In mammals, a single gene encodes prosomatostatin (Goodman *et al.*, 1980; Montminy *et al.*, 1984). This precursor is processed by proteolytic enzymes at mono- and dibasic amino acid residues to release SRIF and SRIF-28 (Sevarino *et al.*, 1987). In some tissues, SRIF is the predominant product of prosomatostatin processing whereas in other tissues it is SRIF-28 suggesting that differential processing of prosomatostatin occurs.

SRIF induces its biological actions by interacting with membrane associated receptors. Those receptors are linked to multiple cellular effector systems. SRIF has been reported to inhibit adenylyl cyclase activity (Jakobs *et al.*, 1983; Law *et al.*, 1991) and voltage dependent Ca^{++} currents (Ikeda and Schofield, 1989; Meriney *et al.*, 1994; Shaprio and Hille, 1993; Wang *et al.*, 1990a) and to potentiate both inwardly and delayed rectifying K^+ currents (Mihara *et al.*, 1987; Wang *et al.*, 1989). SRIF has also been shown to stimulate a protein tyrosine phosphatase and this action has been linked to the antiproliferative actions of SRIF (Buscail *et al.*, 1994, 1995; Liebow *et al.*, 1989; Pan *et al.*, 1992; Tahiri-Joutic *et al.*, 1992).

The SRIF receptors are coupled to distinct cellular effector systems via GTP binding regulatory proteins (G proteins) (Law *et al.*, 1991; Kleuss *et al.*, 1991). A family of G proteins that have been shown to associate with SRIF receptors are the pertussis toxin sensitive G_i and G_o. The G proteins consist of three subunits, alpha (G_a), beta (G_b) and gamma (Gilman, 1987). There are three distinct forms of G_{ia} referred to as G_{ia1}, G_{ia2} and G_{ia3}. These three forms are encoded by different genes but are from 85–94% identical in amino acid sequence (Jones and Reed, 1987). Two forms of G_{oa} exist that are derived from differential splicing and which vary in amino acid sequence in their C-terminal region. Biochemical studies indicate that SRIF receptors interact with multiple pertussis toxin sensitive G proteins (Law *et al.*, 1991, 1993, 1994; Murray-Whelan and Schlegel, 1992; Brown and Schonbrunn, 1993) and functional studies suggest that selective G proteins link SRIF receptors to distinct cellular effector systems (Kleuss *et al.*, 1991; Sengoles, 1994; Tallent and Reisine, 1992). These findings suggest that subtle differences in physical interaction of SRIF receptors with G proteins may direct the receptor to couple to specific cellular effector systems. These subtle

physical interactions may form the molecular basis for the diversity of cellular actions of SRIF.

SRIF has been shown to initiate its biological actions by binding to a family of receptors. Both biochemical and functional studies have suggested the existence of multiple SRIF receptors (for review see Rens-Domiano and Reisine, 1992). Studies in the late 1970's and early 1980's suggested that SRIF and SRIF 28 might be able to induce distinct effects on pancreatic hormone secretion (Brown *et al.*, 1977; Mandarino *et al.*, 1981). SRIF 28 was found to be significantly more potent than SRIF in blocking insulin secretion whereas the reverse potency ratio was observed for inhibition of glucagon release. Receptor binding studies have also suggested that radiolabeled SRIF and SRIF 28 ligands may bind differently in brain versus pituitary tissues (Srikant and Patel, 1981). Furthermore, electrophysiological studies found that SRIF could potentiate a delayed rectifying K^+ current in embroyonic brain neurons in culture whereas SRIF 28 reduced this same current (Wang *et al.*, 1989). Since no cross-desensitization of these responses were observed (Wang *et al.*, 1990b), it has been hypothesized that the two peptides acted on different receptors to modulate the K^+ current.

Biochemical studies have also indicated that subtypes of SRIF receptors are expressed in the body. Using a SRIF affinity column, He *et al.* (1989) was able to purify a 60 kDa SRIF binding protein from rat brain. Antibodies generated against this material immunoprecipitated specific SRIF binding sites from brain and a cell line AtT-20 and specifically immunoprecipitated a 60 kDa ^{35}S-methionine labeled protein from these cells (Theveniau *et al.*, 1992). Furthermore, by immunoblotting, 60 kDa proteins were detected in tissues expressing SRIF receptors but not in tissues lacking any SRIF binding sites. Because only SRIF agonists were available and high affinity SRIF binding to receptors is dependent on G protein coupling, extensive pharmacological analysis on the 60 kDa protein was not possible.

Other investigators have identified 90 kDa SRIF receptors (reviewed in Rens-Domiano and Reisine, 1992). The size of the protein was similar to 90 kDa proteins covalently crosslinked with radiolabeled SRIF ligands. These proteins were found to be heavily glycosylated. Therefore, it was not clearly established whether the differences in size of these proteins and the rat brain SRIF binding site was due to differences in amino acid sequence or oligosacchride composition.

Pharmacological studies have also suggested the existence of SRIF receptor subtypes. Initial studies revealed that the stable SRIF analog SMS 201-955 interacted with more than one site in brain membrane, whereas it appeared to bind to only one site in the pituitary (Tran *et al.*, 1985; Reubi, 1984). Further studies with the stable SRIF analog MK 678, which is the smallest peptide that can bind to SRIF receptors (Veber *et al.*, 1984; Huany *et al.*, 1992), showed a population of sites in brain and cell lines that exhibited high affinity for the peptide and a second population that exhibited no affinity for the peptide (Raynor and Reisine, 1989). Since MK 678 could be iodinated, its high affinity binding sites could be accurately analyzed and its distribution in brain could be determined by autoradiography (Martin *et al.*, 1991). Such studies revealed that the MK 678 sensitive and insensitive sites had distinct distributions in brain, their

drug specificites clearly differed and their ionic and GTP sensitivities varied dramatically (Raynor *et al.*, 1991; Raynor and Reisine, 1992). Thus, the accumulated evidence up until 1991 was that distinct populations of SRIF receptors were expressed in the body. However, clear approaches to identify these sites had not been established.

CLONING OF SRIF RECEPTORS

A major breakthrough in the SRIF field occurred with the cloning of SRIF receptors (Bell and Reisine, 1993). The first two receptors cloned were referred to as SSTR1 and SSTR2 (Yamada *et al.*, 1992). They exhibited approximately 50% amino acid sequence similarity among themselves but little similarity with any other receptors. When expressed in COS cells they were found to bind radiolabeled SRIF analogs with high affinity and in a specific manner. Using probes derived from SSTR1 and SSTR2, Yasuda *et al.* (1992) was able to clone a third receptor, SSTR3. While having high overall amino acid sequence similarity with SSTR1 and SSTR2, SSTR3 had some unique properties. Its third intracellular loop was 10 amino acids larger than either SSTR1 or SSTR2. This is of interest since the size of the third intracellular loops of SSTR1 and SSTR2 are relatively small consisting of 28 amino acids. This difference in size of the third intracellular loop may have functional importance since this is a region of membrane associated receptors that has been proposed to be involved in the coupling to G proteins and celluar effector system.

Following the cloning of SSTR3, two other SRIF receptor subtypes were cloned at about the same time. The receptor that is now referred to as SSTR4 was cloned by Bruno *et al.* (1992) and SSTR5 was cloned by O'Carroll *et al.* (1992). SSTR4 has relatively high amino acid sequence similarity with SSTR1 and has been shown to have similar pharmacological properties with this receptor. In contrast, SSTR5 has relatively high amino acid sequence similarity with SSTR2 and shares its ligand selectivities most closely with SSTR2 than any other SRIF receptor. Because of the similarities in amino acid sequence and pharmacological properties, Hoyer *et al.* (1994) have proposed that subfamilies of SRIF receptor subtypes exist with SSTR1 and SSTR4 comprising one family and SSTR2, SSTR3 and SSTR5 forming the other family.

Since the initial cloning of SSTR1 and SSTR2, the species homologs of each receptor have been identified (Corness *et al.*, 1993; Demchyshyn *et al.*, 1993; Kluxen *et al.*, 1992; Matsumoto, *et al.*, 1994; Meyerhof *et al.*, 1992; O'Carroll *et al.*, 1994; Panetta *et al.*, 1994; Rohrer *et al.*, 1993; Xu *et al.*, 1993; Yamada *et al.*, 1992,1993a,b; Yasuda *et al.*, 1993). Relatively little difference in amino acid sequences of rodent and human forms of SSTR1–SSTR4 have been identified and few differences in pharmacological properties have been reported, suggesting little deviation in the characteristics of these receptors during evolution. In contrast, rat and human SSTR5 are only 81% identical in amino acid sequence and have been reported in one study to exhibit considerable differences in ligand selectivity (O'Carroll et al, 1994).

DISTRIBUTION OF SRIF RECEPTOR mRNAs

The mRNAs for the SRIF receptors have distinct but overlapping distributions in the body (Breder *et al.*, 1992; Bruno *et al.*, 1993; Kaupman *et al.*, 1993; Kong *et al.*, 1994a; Meyerhof *et al.*, 1992; Perez *et al.*, 1994; Wulfsen *et al.*, 1993). All are expressed in brain, but SSTR5 mRNA is very rare in this tissue and is only detectable with sensitive polymerase chain reaction technology (Bruno *et al.*, 1993). All are expressed in the pituitary and gut. In rat SSTR2 mRNA is the only SRIF receptor mRNA detected in the adrenal gland (Kong *et al.*, 1994a) and in human SSTR5 mRNA is the only one expressed in heart (O'Carroll *et al.*, 1994). This latter finding is of interest since recent pharmacological studies in the guinea pig heart have shown that SRIF-28 has a negative inotropic effect on heart and the pharmacological characterization of this response is consistent with the expression of a SSTR5-like receptor (Feniuk *et al.*, 1993). Pancreatic islets predominantly express SSTR3 mRNA (Yamada *et al.*, 1993), although mRNA for the other receptors is also expressed in this tissue. Interestingly, there is little evidence for a functional role of SSTR3 in the pancreas. The high level of SSTR3 mRNA may therefore be untranslated, as has been suggested to occur with the very high level of SSTR3 mRNA in the rat cerebellum (Kong *et al.*, 1994a), a brain region with few if any SRIF receptors (Epelbaum, 1986).

SRIF receptor mRNAs have been found to be highly expressed in tumors (Buscail *et al.*, 1994; Greeman and Melmed, 1994; Kubota *et al.*, 1994; Patel *et al.*, 1994; Reubi *et al.*, 1994, Taylor *et al.*, 1994) which may be related to the usefulness of SRIF analogs in the treatment of cancer (Lamberts *et al.*, 1991). In particular, SSTR2 mRNA is found in most cancerous tissues, which may explain the therapeutic effectiveness of Sandostatin, a compound that selectively interacts with human SSTR2 (O'Carroll *et al.*, 1994).

PHARMACOLOGICAL PROPERTIES OF THE CLONED SRIF RECEPTORS

Ligand Selectivity

The ligand selectivities of each cloned SRIF receptor have been investigated using receptor binding approaches. For these studies, the rodent or human receptors have been expressed in CHO or COS cells so that each receptor can be studied independently and under conditions of known receptor density. Human SSTR1 has been reported to bind SRIF and SRIF-28 with subnanomolar affinities (Raynor *et al.*, 1993a; Yamada *et al.*, 1992; Rens-Domiano *et al.*, 1992). However, most synthetic analogs of SRIF have very low affinity for SSTR1 and no compound has been identified that binds selectively to this receptor (Raynor *et al.*, 1993a).

Mouse SSTR2 also has high and similar affinity for the native SRIFs but also exhibits high affinity for synthetic hexa- and octapeptide analogs including the analogs SMS-201-995, MK 678 and BIM23014 (Raynor *et al.*, 1993a). None of

these compounds bind specifically to SSTR2 although MK 678 preferentially labels this receptor. The analogs NC4-28B and NC8-12 do selectively interact with SSTR2 and have been employed to selectively activate this receptor (Raynor *et al.*, 1993a, b).

SSTR3 has intermediate ligand selectivities between SSTR1 and SSTR2. SRIF and SRIF-28 both bind potently to SSTR3 (Yasuda *et al.*, 1992; Yamada *et al.*, 1993; Raynor *et al.*, 1993a). Unlike SSTR1, synthetic hexa- and octapeptide analogs of SRIF do bind to SSTR3, although at lower potency than they interact with SSTR2. The linear peptide BIM23056 interacts with high affinity with SSTR3 and has some selectivity for this receptor subtype (Raynor *et al.*, 1993a). However, no other compounds have been found to bind selectively to this receptor.

SSTR4, like SSTR1 has high affinity for native SRIF peptides but low affinity for smaller, more rigid analogs (Raynor *et al.*, 1993b). The similar ligand selectivities may be related to the high amino acid sequence similarity of these receptor subtypes which is 61%. The similarities in amino acid sequence and ligand binding properties suggest that the ligand binding pockets or ligand recognition sites of the two receptors may be alike.

SSTR5 is unique among the SRIF receptors since it expresses significantly higher affinity for SRIF-28 than SRIF (O'Carroll *et al.*, 1992). Rat SSTR5 expresses many similar pharmacological characteristics as SSTR2. It has high affinity for both hexa- and octapeptide SRIF analogs, particularly for SMS 201-995 (Raynor *et al.*, 1993a,b). Several peptides have been found to selectively bind to the rat SSTR5 including the linear peptide BIM 23052 and the cyclic compound L362,855 (Raynor *et al.*, 1993b). BIM23052 has recently been used to identify selective functions of rSSTR5 (Rossowski *et al.*, 1993, 1994).

The human homolog of SSTR5 has only 81% amino acid sequence identity with the rat receptor (O'Carroll *et al.*, 1994). SSTR5 is the only SRIF receptor with species variations in amino acid sequence. The amino acid sequence differences may contribute to functional variations of the species homologs since human SSTR5 has much lower affinity for most hexa- and octapeptide analogs of SRIF than the rat receptor. The clinically used analog SMS-201-995, while exhibiting similar high affinity for rat SSTR2 and SSTR5 has much lower affinity for human SSTR5 than human SSTR2, indicating the compound is likely to be a selective SSTR2 agonist in humans (Raynor *et al.*, 1993a,b; O'Carroll *et al.*, 1994). Similarly, the peptide BIM 23052, which selectively interacts with rat SSTR5 and has been employed to identify selective functions of SSTR5 in studies using rats, is not selective for human SSTR5 and therefore would not be useful as a selective SSTR5 agonist in humans (O'Carroll *et al.*, 1994). The only compound that exhibits some selectivity for rat and human SSTR5 compared to the other SRIF receptor subtypes is L362,855 (O'Carroll *et al.*, 1994).

Distinct Biological Functions of SRIF Receptor Subtypes

Using the subtype selective agonists at the different cloned SRIF receptors has allowed for the determination of the distinct functions mediated by SRIF

receptor subtypes in the body. Initial studies have shown that the rank order of potencies of SRIF analogs to bind to SSTR2 correlated highly with their potencies to inhibit growth hormone secretion from rat anterior pituitary cells in culture (Raynor *et al.*, 1993a,b). In contrast, there was no relationship between the potencies of compounds to interact with the other SRIF receptor subtypes and their potencies to modulate growth hormone secretion. These findings suggest that SSTR2 may selectively mediate SRIF inhibition of growth hormone release from the anterior pituitary.

Rossowski and Coy (1993) reported that the SSTR5 selective agonist BIM23052 inhibited in vivo insulin release in the rat as potently as SRIF. However, SSTR2 and SSTR3 selective agonists were either much less potent or ineffective in reducing insulin secretion. These authors have proposed the SSTR5 in the rat may selectively mediate the inhibition of insulin release by SRIF.

In contrast, these same authors (Rossowski and Coy, 1994) have proposed that SSTR2 may be selectively involved in the control of glucagon secretion. They reported that two SSTR2 selective agonists, NC-8-12 and DC-25-100 were more potent than SRIF in reducing glucagon secretion in vivo whereas an SSTR5 selective agonist was much less potent than SRIF and SSTR3 selective agonists were ineffective in modulating glucagon release. These findings indicate that glucagon and insulin secretion may be under the control of different SRIF receptor subtypes, which is consistent with previous evidence suggesting that separate SRIF 28-preferring and SRIF-preferring receptors were involved in the control of the secretion of these hormones (Mandarino *et al.*, 1981; Brown *et al.*, 1977).

In other studies by Rossowski *et al.* (1994), both SSTR2 and SSTR5 selective agonist were potent inhibitors of bombesin-stimulated pancreatic amylase secretion in vivo suggesting that these receptors had a role in the regulation of other pancreatic secretions. In contrast, it was observed that SSTR2-selective agonists inhibited gastric acid secretion much more potently that either SSTR3 or SSTR5 selective agonists suggesting that this function of SRIF was only mediated by SSTR2. These in vivo studies are striking and suggest that it may be possible to selectively regulate distinct endocrine and exocrine functions of SRIF. As a result, subtype selective agonists may have a number of clinical applications in the treatment of disorders of hypersecretion of metabolic hormones such as insulin and glucagon and diseases of the gastrointestinal tract associated with abnormal acid or amylase release.

MOLECULAR AND CELLULAR ACTIONS OF SRIF

SRIF Receptor/G Protein Coupling

SRIF receptors have been shown to couple to G proteins using a number of different approaches. High affinity agonist binding to SRIF receptors in brain, peripheral organs and in cell lines is reduced by GTP analogs and pertussis toxin

treatment indicating an interaction of the receptors with either G_i and/or G_o (Epelbaum, 1986; Raynor and Reisine, 1992; Law *et al.*, in press). Multiple approaches have been used to identify which G proteins associate with SRIF receptors. Law et al (1991) solubilized SRIF receptor/G protein complexes with the mild detergent CHAPS and was able to immunoprecipitate rat brain and AtT-20 SRIF receptors with peptide directed antisera against G_{ia1}, G_{ia3} and G_{oa} but not antisera against G_{ia2}, suggesting SRIF can selectivity interact with some but not all pertussis toxin sensitive G proteins. Similar results were observed by Murray-Whelan and Schelgel (1992) and Brown and Schonbrunn (1993) using complementary approaches. The different G proteins may couple SRIF receptors to distinct cellular effector systems. Tallent and Reisine (1992) showed that G_{ia1} selectively coupled SRIF receptors to adenylyl cyclase in AtT-20 cells. In these studies, peptide directed antisera against G_{ia1} blocked the inhibition of forskolin stimulated adenylyl cyclase activity by SRIF. In contrast, antisera against G_{ia2}, G_{ia3} and G_{oa} did not alter the ability of SRIF to inhibit adenylyl cyclase activity. Similar results have also been reported by Sengoles (1994). She generated GH_3 cells expressing pertussis toxin insensitive G proteins. Pertussis toxin treated cells expressing a mutant G_{ia1} responded to SRIF with an inhibition of cAMP formation, whereas cells expressing a mutant G_{ia2} did not. However, this author also reported that G_{ia3} coupled SRIF receptors to adenylyl cyclase, indicating that both G_{ia1} and G_{ia3} couple SRIF receptors to adenylyl cyclase.

While G_{ia} couples SRIF receptors to adenylyl cyclase, Kleuss *et al.* (1991) have proposed that G_{oa} selectively couples SRIF receptors to Ca^{++} channels. In their studies, G_{oa} expression was knocked-down using antisense approaches and coupling of the SRIF receptor with Ca^{++} channels in GH_3 was diminished. In contrast, knock-down of G_{ia} had no clear effect on SRIF receptor coupling to the same channel. These investigators have also proposed that the $beta_{36}$ and the $gamma_3$ subunits of G proteins are necessary for SRIF receptors to couple to Ca^{++} channels (Kleuss *et al.*, 1992, 1993). These findings are consistent with previous biochemical studies of Law *et al.* (1991) who reported that the $beta_{36}$ and the $gamma_3$ subunits selectively form stable complexes with SRIF receptors.

To investigate the G proteins associated with the cloned SRIF receptors, Law *et al.* (1993), solubilized SSTR2A expressed in CHO-DG44 cells and attempted to immunoprecipitate SSTR2A/G protein complexes with peptide-directed antisera against different alpha subunits. These investigators reported that G_{ia3} and G_{oa} formed stable complexes with SSTR2A. This finding differed from results obtained with SRIF receptors expressed in brain or AtT-20 cells which indicated that G_{ia1}, as well as G_{ia3} and G_{oa} associate with SRIF receptors (Law *et al.*, 1991). The difference in the results may be due to the detection of multiple SRIF receptors in brain and AtT-20 cells, so that a mixture of SRIF receptor subtypes were detected whereas the studies with the cloned SSTR2A only examined one receptor subtype. Alternatively, processing or physical modifications of endogenous SSTR2-like receptors may direct that receptor to associate with G_{ia1}, whereas the unprocessed cloned receptor is unable to effectively associate with this G protein.

The functional significance of the lack of coupling of SSTR2A to G_{ia1} is not

established. It may be related to the inability of some investigators to observe clear coupling of this cloned receptor to adenylyl cyclase (Bell and Reisine, 1993; Buscail *et al.*, 1994; Law *et al.*, 1993; Rens-Domiano *et al.*, 1992; Yasuda *et al.* 1992). However, it should be noted that other investigators have found this receptor mediates the inhibition of cAMP formation by SRIF (Garcia and Myers, 1994; Hershberger *et al.*, 1994; Hoyer *et al.*, 1994; Kaupmann *et al.*, 1993; Tomura *et al.*, 1994).

SSTR3 associates with pertussis toxin sensitive G proteins which couple the receptor to adenylyl cyclase (Yasuda *et al.*, 1993). SSTR3 expressed in CHO-DG44 cells which lack G_{ia1} and G_{ia2} but express G_{ia3}, did not couple to adenylyl cyclase. In contrast, SSTR3 expressed in COS-7 cells, which express all three forms of G_{ia}, and 293 cells which express G_{ia1} and G_{ia3}, but not G_{ia2}, coupled to adenylyl cyclase and mediated the inhibition of cAMP formation by SRIF (Law *et al.*, 1993). These findings suggested that G_{ia1} was necessary to couple SSTR3 to adenylyl cyclase (Law *et al.*, 1994; Yasuda *et al.*, 1992). To more directly test this hypothesis, Law *et al.* (1994) cotransfected SSTR3 with either G_{ia1} or G_{ia2} into CHO-DG44 cells. In cells expressing G_{ia1}, SSTR3 coupled to adenylyl cyclase and mediated SRIF inhibition of cAMP accumulation whereas in cells expressing G_{ia2}, but lacking G_{ia1}, it did not. These findings established that G_{ia1} couples SSTR3 to adenylyl cyclase.

Identification of functional differences of G_{ia} subtypes is particularly interesting since the amino acid sequences of the alpha subunits are very similar. G_{ia1}, which couples SSTR3 to adenylyl cyclase and G_{ia3}, which does not, are 94% identical in amino acid sequence (Jones and Reed, 1987). This high similarity indicates that small structural differences of the alpha subunits must be responsible for the functional differences.

To identify regions of G_{ia} involved in coupling SSTR3 to adenylyl cyclase, Law *et al.* (1994) generated chimeric alpha subunits consisting of the N-terminal two-thirds of G_{ia2} ligated to the C-terminal third of either G_{ia1} or G_{ia3}. Following coexpression of SSTR3 with these individual chimeras in CHO-DG44 cells, SSTR3 was found to couple to adenylyl cyclase in cells expressing the chimera consisting of the N-terminus of G_{ia2} and the C-terminus of G_{ia1} but not G_{ia3}. This finding revealed an essential role of the C-terminus of G_{ia1} in coupling SSTR3 to adenylyl cyclase, which is of importance since this region of the G_{ia} subtypes only differ by a few amino acid residues. As a result, only a few amino acid residues are critical for directing SSTR3 to couple to adenylyl cyclase.

Coupling of SRIF Receptors to Ionic Conductance Channels

SRIF receptors couple to multiple ionic conductance channels to modulate the functions of electrically active cells. SRIF inhibits voltage dependent, L-type Ca^{++} channels to reduce Ca^{++} conductance and influx into cells (Ikeda and Schofield, 1989; Kleuss *et al.*, 1991; Wang *et al.*, 1990a). Furthermore, SRIF potentiates K^+ conductance through inwardly rectifying, delayed rectifying, Ca^{++}-dependent and an M-type K^+ channels (Mihara *et al.*, 1987; Moore *et al.*,

1988; Wang *et al.*, 1989; White *et al.*, 1991). The regulation of Ca^{++} and K^{+} conductances may be related to the ability of SRIF to generally inhibit neurotransmitter and hormone release and reduce the firing activity of neurons and endocrine and exocrine cells.

The subtypes of receptors which mediate the regulation of these channels by SRIF have not been clearly established. MK 678, an agonist that preferentially binds to SSTR2, has been shown to inhibit Ca^{++} currents in embroyonic brain neurons in culture (Raynor *et al.*, 1991) and in the pituitary cell line AtT-20 (Reisine *et al.*, 1994). Furthermore, other SSTR2 selective agonists have been found to inhibit Ca^{++} currents in AtT-20 cells, suggesting that this receptor subtype is coupled to this current. In these cells, the SSTR5-selective agonist BIM23052 also inhibits the L-type Ca^{++} current, suggesting that this receptor subtype may couple to the Ca^{++} channel.

SRIF Receptors and Cell Proliferation

SRIF analogs have antiproliferative effects and are used clinically to treat tumors expressed in endocrine and exocrine organs (Lamberts *et al.*, 1991). Susini and coworkers have reported that the cloned human SSTR1 and SSTR2 and the rat and human SSTR5 can mediate antiproliferative effects of SRIF and some of its analogs, whereas SSTR3 and SSTR4 do not appear to be involved in mediating SRIF's effects on cell proliferation (Buscail *et al.*, 1994, 1995). The ability of SSTR1 and SSTR2 to mediate antiproliferative effects of SRIF were correlated with their abilities to mediate the stimulation of a membrane tyrosine phosphatase. The effects of SRIF were not related to actions on adenylyl cyclase or an inhibition of cAMP formation because neither receptor coupled to adenylyl cyclase in the cells examined. The close association of a tyrosine phosphatase with SSTR2 was further established by the coimmunoprecipitation of the receptor and phosphatase with antisera against the receptor or enzyme (Zeggari *et al.*, 1994). The coupling of SSTR2 to the phosphatase was GTP and pertussis toxin-sensitive suggesting an intermediary role of a G protein in the coupling. However, the identity of the G protein (s) has not been established. Furthermore, it is not clearly established what is the mechanism by which stimulation of the tyrosine phosphatase causes antiproliferative effects of SRIF.

SSTR5 was also able to mediate antiproliferative effects of the SRIF analog RC-160 (Buscail *et al.*, 1995). However, no coupling of this receptor with tyrosine phosphatase activity was observed and pharmacological treatments which block tyrosine phosphatase activity did not prevent the antiproliferative effects of SRIF in cells expressing this receptor. In contrast, in CHO cells expressing this receptor, SRIF was able to inhibit Ca^{++} mobilization induced by cholecystokinin. Conceivably by blocking Ca^{++} mobilization, SRIF may hinder activation of key enzymes involved in the cell proliferation process.

These findings indicate that three different receptor subtypes are involved in mediating antiproliferative effects of SRIF analogs. The most commonly employed SRIF analog used to treat tumors in humans is Sandostatin (Lamberts *et al.*, 1991), which selectively interacts with human SSTR2 (O'Carroll *et al.*,

1994). The finding that human SSTR1 and SSTR5 also mediate antiproliferative effects of SRIF analogs and that mRNA for these receptors are also detectable in human tumors, suggest that agonists selective for these receptors may be useful in treating cancers. Agonists selective at SSTR1 may be particularly useful since continuous stimulation of SSTR2 with agonist causes rapid desensitization (Rens-Domiano *et al.*, 1992) which could result in loss of the theraputic effects of Sandostatin thereby diminishing its clinical effectiveness. Similarly, SSTR5 rapidly desensitizes (Raynor *et al.*, 1993b). SSTR1, however, is the one SRIF receptor subtype that is very resistent to agonist induced regulation (Rens-Domiano *et al.*, 1992) and therefore activation of this receptor subtype could provide lasting inhibition of tumor growth.

STRUCTURE–FUNCTION ANALYSIS OF THE SRIF RECEPTORS

With the cloning of the SRIF receptor, it has now become possible to investigate structural aspects of the receptors involved in their ligand recognition and coupling to cellular effector systems. Many G protein linked receptor have conserved aspartate residues in their second transmembrane spanning region that are involved in mediating Na + regulation of agonist binding and are also believed to have a role in G protein coupling of the receptors. Mutagenesis studies of alpha2-adrenergic receptors (Horstman *et al.*, 1990) and delta opioid receptors (Kong *et al.*, 1993a) have revealed a critical role of this conserved charged amino acid in Na + regulation of agonist binding. Mutations of aspartate 79 of the mouse SSTR2 to an asparagine was also found to abolish Na + regulation of agonist binding to this receptor (Kong *et al.*, 1993b). However, the ligand selectivities of this receptor were similar to the wild-type receptor. This finding differs from those observed with the delta opioid receptor, which showed a critical role of the conserved aspartate in the affinity of the receptor for agonists as well as the sensitivity of the receptor to Na + regulation.

To identify ligand binding domains of the SRIF receptors, Fitzpatrick and Vandlen (1994) constructed a series of chimeric receptors consisting of fragments of SSTR1 and SSTR2. They labeled the receptors with an iodinated SRIF-28 ligand which binds to both SRIF receptor subtypes equally well and then tested MK 678 for its ability to displace the binding of this radioligand to the different mutant receptors. MK 678 is the smallest, most rigid SRIF analog available that binds potently to SSTR2 (Veber *et al.* 1984; Huany *et al.*, 1992) but does not interact with SSTR1. By screening different SSTR1 chimeras containing small fragments of SSTR2 for their affinities for MK 678, it was possible to identify MK 678 binding domains. These authors showed that both the second and third extracellular loops contributed to MK 678 binding with the third extracellular loop being most important for the interaction of this synthetic peptide with SSTR2.

This finding is particularly important because it emphasizes the importance of extracellular loops in the binding of peptides to their receptors. Initial mutagenesis studies on the beta-adrenergic receptor indicated that catecholamines and

synthetic adrenergic ligands bound to hydrophobic pockets created by the transmembrane spanning regions of the receptor and that hydrophillic extracellular loops of the receptor contributed little to ligand binding (Dixon *et al.*, 1987). Such findings have served as the model to explain how ligands interact with G protein linked receptors. However, different mechanisms may subserve peptide interactions with their receptors. Peptides are larger molecules than catecholamines or classical neurotransmitters and may therefore not easily fit into a hydrophobic pocket. Studies with the various peptide receptors such as the kappa opioid receptor (Kong *et al.*, 1994) as well as the tachykinin receptors (Gether *et al.*, 1993) and SSTR2 (Fitzpatrick and Vandlen, 1994) have established the critical role of hydrophillic extracellular loops as having a predominant role in ligand binding. Furthermore, because of the relatively large size of the peptides, multiple contact sites appear to be required for binding to peptide receptors. This has been reported for the tachykinin receptors and is likely to be the case for SRIF receptors since the studies of Fitzpatrick and Vandlen (1994) have shown that SRIF-28 binding to SSTR2 requires additional sites besides the second and third extracellular loops.

SSTR5, unlike the other SRIF receptors, has significantly higher affinity for SRIF-28 than SRIF (O'Carroll *et al.*, 1992). To investigate the structural basis of this selectivity, Ozenberger and Hadcock (1995) generated a series of chimeric receptors consisting of fragments of SSTR2 and SSTR5. SSTR2 has similar high affinity for SRIF-28 and SRIF. Their analysis of the chimeras lead them to propose that a region in the vicinity of transmembrane 6 contains sites in SSTR2 that were necessary for high affinity binding of SRIF that were not present in SSTR5. They then observed that there is a conserved tyrosine in transmembrane 6 of SSTR1-4 that is a phenylalanine in SSTR5. Mutation of the phenylalanine in SSTR5 to a tyrosine confered onto the mutant receptor high affinity for SRIF and the receptor lost its selectivity for SRIF-28. Therefore, a single hydroxyl group was critical for creating the selectivity of SSTR5 for SRIF-28. Interestingly, while this residue was critical for the high affinity of the receptor for SRIF, it is not necessary for high affinity for SRIF-28, which is consistent with the results of Fitzpatrick and Vandlen (1994). SRIF-28 must therefore have more determinants in SRIF receptors for binding than either SRIF or the synthetic SRIF analogs. SRIF-28 is the largest peptide that binds to SRIF receptors and it is conceivable that the N-terminal extension of the peptide allows it to recognize more contact sites in SRIF receptors than the smaller peptides. Since SRIF-28 is the only peptide that binds to all SRIF receptors with high affinity, it is possible that all SRIF receptors have common recognition sites for this extended part of SRIF-28.

Regions of SRIF receptors involved in coupling to G proteins and adenylyl cyclase may involve the third intracellular loop. SSTR2 is expressed as two forms due to alternative splicing (Vanetti *et al.*, 1992, 1993). The non-spliced form SSTR2A and the spliced form SSTR2B, have identical amino acid sequences from residues 1-331. They then diverge in amino acid sequence in their C-terminus with SSTR2B being shorter in length and differing in sequence. SSTR2B more effectively couples to adenylyl cyclase than SSTR2A (Vanetti *et al.*, 1993; Reisine

et al. 1994a). Mutagenesis studies have shown that the difference in coupling are not due to the length of the C-terminus. In fact, truncation of the C-terminus of SSTR2 results in a receptor that effectively couples to adenylyl cyclase (Vanetti *et al.* 1993; Woulfe and Reisine, 1994; Law *et al.*, 1995). Therefore, the C-terminus of SSTR2 is not needed for coupling to adenylyl cyclase. This findings suggests that intracellular loops may be important for coupling the SRIF receptors to adenylyl cyclase.

The importance of intracellular loops of the SRIF receptors in coupling the receptors to adenylyl cyclase is further suggested from studies involving SSTR1. In studies by Reisine *et al.* (1994b), SSTR1 was not able to effectively couple to adenylyl cyclase in COS–7 cells. In contrast, a chimeric SSTR1 containing the third intracellular loop of the delta opioid receptor coupled to adenylyl cyclase and mediated SRIF inhibition of cAMP accumulation. The delta opioid receptor couples to adenylyl cyclase. However, a chimeric delta receptor containing the third intracellular loop of SSTR1 did not effectively couple to adenylyl cyclase. These findings suggest that the inability of SSTR1 to couple with adenylyl cyclase may be due to unique sequences in the third intracellular loop. Conversely, the ability of delta receptor and possibly SSTR2 to couple to adenylyl cyclase may be due to their unique third intracellular loops.

FUTURE DIRECTIONS

The cloning of the SRIF receptors has lead to structure-function analysis which is revealing how SRIF induces its cellular functions. It has also provided the means to identify subtype selective agonists. Such compounds are allowing for investigation into the specific functions of each receptor subtype. They will also lead to the development of a new generation of SRIF drugs. Such drugs could be employed to treat metabolic disorders involving imbalances in insulin and glucagon secretion. They could also be used to treat diseases of the gastrointestinal tract involving abnormalities in acid secretion. In addition, they may be more useful than present strategies to treat tumors or cancers. It may also be possible to develop SRIF ligands that can cross diffusion barriers such as the blood brain barrier. This could be accomplished through molecular modeling of rigid SRIF analogs such as L362,301 (Huany *et al.*, 1992) and the development of non-peptide SRIF agonists. SRIF drugs that could enter the nervous system could also be employed to treat diseases such as epilepsy and Alzheimer's disease, which are associated with deficits of SRIF in the brain (Epelbaum, 1986; Raynor and Reisine, 1992).

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