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Molecular Motifs for Subtype-specific Coupling of Somatostatin Receptors to Inhibitions of the Na-H Exchanger NHE1 And Somatostatin, Acting at Receptor Subtype SSTR1, Inhibits Rho Activity and the Assembly of Actin Stress Fibers

by

Chin-Yu Lin

DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

Oral and Craniofacial Sciences

in the

GRADUATE DIVISION

of the

UNIVERSITY OF CALIFORNIA, SAN FRANCISCO

DEDICATION

To my beautiful wife Hui-Chun, who is always an inspiration of my life.

To my newly born sons Elias and Elijah, whose births are the most wonderful gifts in the holiday season of year 2002.

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Molecular Motifs for Subtype-specific Coupling of Somatostatin Receptors to Inhibition of the Na^+-H^+ Exchanger NHE1 And Somatostatin, Acting at Receptor Subtype SSTR1, Inhibits Rho Activity and the Assembly of Actin Stress Fibers

Chin-Yu Lin

Abstract: The focus of this dissertation is on how the neuroendocrine peptide somatostatin (SST) inhibits activity of the ubiquitously expressed plasma membrane Na^+-H^+ exchanger, NHE1, and how it inhibits dynamic changes in the actin-based cytoskeleton. SST activates a family of five plasma membrane heptahelical receptors (SSTR1-5), a subfamily of the class A group of G protein-coupled receptors (GPCR). The sponsor's laboratory previously determined that SST inhibition of NHE1 is mediated by SSTR1, but not SSTR2. Studies with chimeric SSTR2/SSTR1 receptors found that both intracellular loop 2 and 3 of SSTR1 are required to confer SST inhibition of NHE1. The specific amino acid residues necessary to confer coupling, however, have not been identified. Moreover, whether other SSTR subtypes, in addition to SSTR1, regulate NHE1 has not been determined. Chapter 1 of this dissertation focuses on SST regulation of NHE1, by determining the coupling of SSTR subtypes and the receptor determinants required. Results indicate that SSTR1, 3, and 4, but not SSTR 2 and 5, couple to the inhibition of NHE1, and they identify conserved amino acid motifs shared by SSTR1, 3 and 4 that confer coupling. Moreover, these conserved motifs are found in other GPCR subfamilies linked to the

inhibition of NHE1, which suggests that common motifs shared by distinct GPCRs can be used to regulate a common effector.

Recent work from the sponsor's laboratory has shown that activation of NHE1 by integrin receptors and by GPCR is mediated by Rho GTPase and the Rho kinase ROCK. Moreover, increased NHE1 activity is critical for dynamic changes in actin filament formation and focal adhesion assembly in response to activated integrins and GPCR. Work in the Chapter 2 explores the hypothesis that because SST inhibits NHE1 activity, it might also inhibit cytoskeleton reorganization. Results indicate that SST inhibits integrin- and thrombin-induced Rho activity, actin filament formation, and focal adhesion assembly. Consistent with findings in Chapter 1 that SSTR1, but not SSTR2, couples to the inhibition of NHE1, SSTR1, but not SSTR2, mediates SST attenuation of Rho and cytoskeletal reorganization. These findings are the first to identify a GPCR linked to the inhibition of Rho.

Diane Barber

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CHAPTER I

INTRODUCTION

SOMATOSTATIN

The neurohumoral peptide somatostatin (SST) is widely distributed throughout the central and peripheral nervous systems, the gastrointestinal tract, and a number of major peripheral organs. SST binds to five receptor subtypes (SSTR1-5) to inhibit a number of processes, including hormone secretion, exocrine secretion, cell proliferation, and tumor progression. All somatostatin receptors (SSTRs) are heptahelical G-protein-coupled receptors (GPCR) that bind SST with equal affinity and are linked to the pertussis toxin (PTX)-sensitive inhibition of adenylyl cyclase. Distinct effects of different SSTR subtypes on effectors other than adenylyl cyclase, however, have also been identified and these are mediated by both PTX-sensitive and -insensitive mechanisms.

In most organisms, SST exists in two biologically active forms. It was first isolated from the hypothalamus as a "somatotropin-release inhibiting factor" and characterized as a cyclic peptide of 14 amino acids, SST-14, with a disulfide-bond between cysteine residues at positions 3 and 14 (1). Later, a congener of SST-14 with 14 more amino acids extended at the N-terminus, SST-28, was identified (2). In mammals, SST-14 and SST-28 are derived from a single gene encoding a common preprosomatostatin 116 amino acid precursor (3-6). Preprosomatostatin has a 24 amino acid-long signal peptide at the N-terminus, followed by a proregion, which precedes SST-14 and SST-28 at the C-terminus. Preprosomatostatin is synthesized on the rough endoplasmic reticulum and

directly released into the lumen of the endoplasmic reticulum, where the signal peptide is cleaved co-translationally to generate prosomatostatin (7). Prosomatostatin is subsequently transported to the Golgi apparatus and packaged into secretory vesicles, where bioactive SST-14 and SST-28 are generated by propeptide-converting enzymes, termed convertases. Candidate convertases include PC1, PC2, and PACE4 for generating SST-14 (8,9), and PACE4 and furin for generating SST-28 (9). Unless indicated otherwise, throughout this thesis SST is used synonymously with SST-14.

Tissue Distribution and Functions of Somatostatin

Although originally isolated from the hypothalamus, SST is widely distributed throughout the central and peripheral nervous systems, the gastrointestinal tract, and a number of other organs, including the spleen, the, thymus, the kidney, the adrenal medulla, the thyroid, and the submandibular glands. SST generally acts as an inhibitory regulator of endocrine and exocrine secretions, neurotransmission, and cell proliferation.

In the central nervous system, the highest levels of expression are in the hypothalamus, the limbic system, the mesencephalic region, and the basal ganglia (10-12). In the peripheral nervous system, SST is expressed in both sensory (13-18) and autonomic nerves (19-27). In the central nervous system, SST inhibits the endocrine secretions of corticotrophin-releasing hormone, thyrotrophin-releasing hormone, growth hormone (1) and thyroid stimulating hormone (28,29). SST also regulates the neurotransmission of central and

peripheral nervous systems by inhibiting the release of other neurotransmitters, such as γ-amino-butyric acid, acetylcholine, dopamine, serotonin, and norepinephrine (30-35).

A pathological role for SST in the central nervous system is suggested by the fact that in patients of Alzheimer's disease there is a selective and specific decrease in cortical SST concentration (36-38). Among various peptides present in cortical regions, SST is the only peptide found to consistently be reduced in Alzheimer's disease. The extent of decreased SST correlates with increased severity of the disease, characterized by the loss of function in locomotor activity and cognition and neuronal degeneration.

In the gastrointestinal tract, somatostatin is expressed in epithelial endocrine δ cells of the stomach, small intestine, and large intestines and in neurons of the enteric nervous system (39-42). Somatostatin released from gastric and intestinal epithelia inhibits endocrine secretions of gastrin, pancreozymin, and vasoactive intestinal peptide, motilin, gastric inhibitory peptide, and secretin (43). It also inhibits exocrine secretions of gastric acid, pepsin, bile, and colonic fluid (43). Somatostatin released from enteric neurons in both submucosal and myenteric plexuses primarily inhibits smooth muscle contractility, and hence gastrointestinal motility (42). SST is also expressed in and released from δ cells in pancreatic islets (39,44-46), where it SST inhibits glucagon release from α cells, insulin release from β cells, and the release of endogenous SST from δ cells (47).

Somatostatin is expressed in a number of additional neuroendocrine tissues, including the spleen (48), thymus (49), kidney (50), adrenal medulla (51), thyroid (52,53), and submandibular glands (54,55). SST also inhibits the endocrine and exocrine secretions of these tissues, such as salivary secretion from the submandibular salivary gland (55), the secretion of different growth factors, cytokines and immunoglobulins from lymphocytes (56), rennin from the kidney (57,58), aldosterone and catecholamine from the adrenal gland (51), and T3, T4, and calcitonin from the thyroid gland (52,53).

The Role of Somatostatin in Cell Proliferation and Tumor Progression

Somaotstatin has an established inhibitory effect on cell proliferation. It inhibits the proliferation of lymphocytes, inflammatory cells, intestinal mucosal cells, precursor cells of cartilage and bone, and tumor cells (59-63). A direct antiproliferative effect of somatostatin occurs through triggering either cytotoxic apoptosis or cytostatic cell cycle arrest (64,65). Additionally, SST indirectly induces apoptosis by activating a caspase-8 mediated intracellular acidification, which leads to mitochondrial dysfunction (66). The cytostatic response to SST is mediated by the activation of tyrosine phosphatases, which leads to the induction of retinoblastoma protein Rb and G1 cell cycle arrest (65).

With inhibitory effects on growth factor secretion and cell proliferation, SST has been suggested to play an important role in the tumorigenesis and cancer progression. High levels of SSTR expression occur in a large number of human tumors, including tumors of neuroendocrine origin, breast tumors, brain

tumors, kidney tumors and lymphomas (67,68). The presence of SSTR on the peritumoral vascular system, which is independent of the presence or absence of SSTR in the tumor, has also been found in several types of malignant neoplasm (69). In animal tumor models, the process of tumor progression can be inhibited by the addition of SST (70,71). Somatostatin inhibits the growth of SSTR-presenting tumors by inducing apoptosis or cell cycle arrest, and indirectly inhibits the release of hormones and growth factors that act to stimulate tumor growth or tumoral angiogenesis (67,69,72,73). Somatostatin also stimulates local vasoconstriction in peritumoral blood vessels, which is suggested to decrease the blood supply to tumors and hence, induce shrinkage (74). Furthermore, SST impairs tumor progression by inhibiting both local and systemic immune responses (56).

Clinically, SST has been used in the diagnosis and treatment of human tumors. Because SST has a very short circulating half-life, synthetic SST analogues with a longer half-life have been developed. One radiolabelled synthetic SST analogue, [111In-DTPA-Dphe1] Octreotide, has been approved as a clinical imaging reagent in somatostatin receptor scintigraphy. Somatostatin receptor scintigraphy is a sensitive, non-invasive tool for imaging SSTR-expressing tumors, such as neuroendocrine tumors, insulinomas, gastriomas, and some breast cancers (75-80). The major clinical application of somatostatin receptor scintigraphy today is the detection and staging of gastrointestinal neuroendocrine tumors. Because of their anti-neoplastic effects, SST and its

SOMATOSTATIN RECEPTORS

The general inhibitory action of SST is mediated through its interaction with five identified receptor subtypes, SSTR1-5, encoded by five distinct genes (83-90). The SSTR2 gene encodes alternatively spliced variants, SSTR2A and SSTR2B. The only difference between these splice variants is a 23 amino acid extension at the C-terminus of SSTR2A (91,92). SSTR can form homodimers with other SSTR subtypes and heterodimers with other G protein-coupled receptors, such as the dopamine D2 receptor (93,94). The functional significance of SSTR dimerization, however, is currently unknown.

Hydrophobicity analyses indicate that all SSTRs share a similar topology typical of GPCR, including seven hydrophobic segments that likely form transmembrane spanning α-helices and a carboxyl-terminal cytoplasmic domain (95). The overall amino acid identity among the SSTRs is at least 46% (65% similarity), with the highest level of identity found in the transmembrane-spanning regions and the highest divergence at the amino- and carboxyl-termini (96). Conserved among all SSTRs is a sequencece motif, YANSCANPI/VLY, in the seventh transmembrane region that is unique to the SSTR family. Phylogenetic analysis based on amino acid sequences indicates that SSTR can be divided into two subgroups; one comprising SSTR1 and 4, the other SSTR2, 3, and 5 (97). The fidelity of this sequence-based subgrouping, however, does not extend to shared intracellular signaling mechanisms.

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Tissue Distributions of Somatostatin Receptors

Distinct patterns of SSTR expression are found in the central nervous system, the gastrointestinal tract, and many tumors. SSTR2 generally has the widest tissue distribution, and all five receptor subtypes co-exist in many tissues. Because subtype-specific antibodies for all five SSTR have not been developed, studies on tissue distribution have relied on determining SSTR mRNA expression. Although transcripts for all 5 SSTR are found in the central nervous system, SSTR1 and SSTR2 are the predominant subtypes and are widely expressed in the cerebral cortex, the hippocampus, the amygdala, and the hypothalamus (98). SSTR3 expression is restricted to the cerebellum (98,99). In the gastrointestinal tract, all 5 SSTR are expressed in villus cells of the epithelium, in smooth muscle cells of the external longitudinal muscle layer, and in neurons within the submucosal plexus. SSTR1, 2, and 3, however, are the only subtypes expressed in neurons within the myenteric plexus (98,100,101). In pancreatic islets, glucagon-secreting α cells express predominatly SSTR2, insulin-secreting β cells express predominantly SSTR1 and 5, and SST-secreting δ cells express predominantly SSTR5 (102).

In addition to normal tissues, most neuroendocrine tumors express high levels of SSTR and a single tumor usually expresses more than one receptor subtype. Many tumor cell lines of pituitary, islet, pancreatic, breast, neural, and hematopoietic origins express multiple subtypes of SSTR (100,103-105). In many human tumors, such as pituitary tumors, carcinoid tumors, insulinomas, glucagonomas, pheochromocytomas, breast carcinomas, renal carcinoma,

prostate carcinoma, meningioma, and glioma, multiple subtypes of SSTR are expressed with SSTR2, 1,and 5 being the most abundant (69,106-108). In addition to the expression of SSTR in tumor cells, the presence of SSTR-positive blood vessels has been identified in the peritumoral zone of several types of malignant tumors (69). The expression of SSTR in the tumors and the surrounding vascular tissues suggests a possible role for SST in carcinogenesis.

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INTRACELLULAR SIGNALING OF SOMATOSTATIN RECEPTORS

In most cell types, the general inhibitory effect of SSTR activation on cell functions is mediated through both pertussis toxin (PTX)-sensitive and -insensitive signaling mechanisms. The PTX-sensitive mechanisms of SSTRs are mediated by coupling through α_i subunits of heterotrimeric G proteins, consistent with the selective inactivation of these GTPases by PTX. In contrast, although PTX-insensitive signaling by SSTRs is mediated by a GTP-dependent (109), the specific α subunit or G protein family involved remains unknown. The PTX-sensitive signaling pathways of SSTRs include the inhibition of adenylyl cyclase (109-112) (Fig. 1), the activation of K^+ channels (113-115) (Fig. 1), the inhibition of voltage-dependent Ca^{2+} channels (116,117) (Fig. 1), cell cycle arrest (65), and apoptosis (64). SST activation activation of protein phosphatases has been found to occur through PTX-sensitive and –insensitive mechanisms (118-125) (Fig. 1). In contrast, inhibition of the Na^+-H^+ exchanger NHE1 (109,126,127) (Fig. 1), and the phosphorylation-dependent desensitization of SSTRs (128) are exclusively PTX-insensitive.

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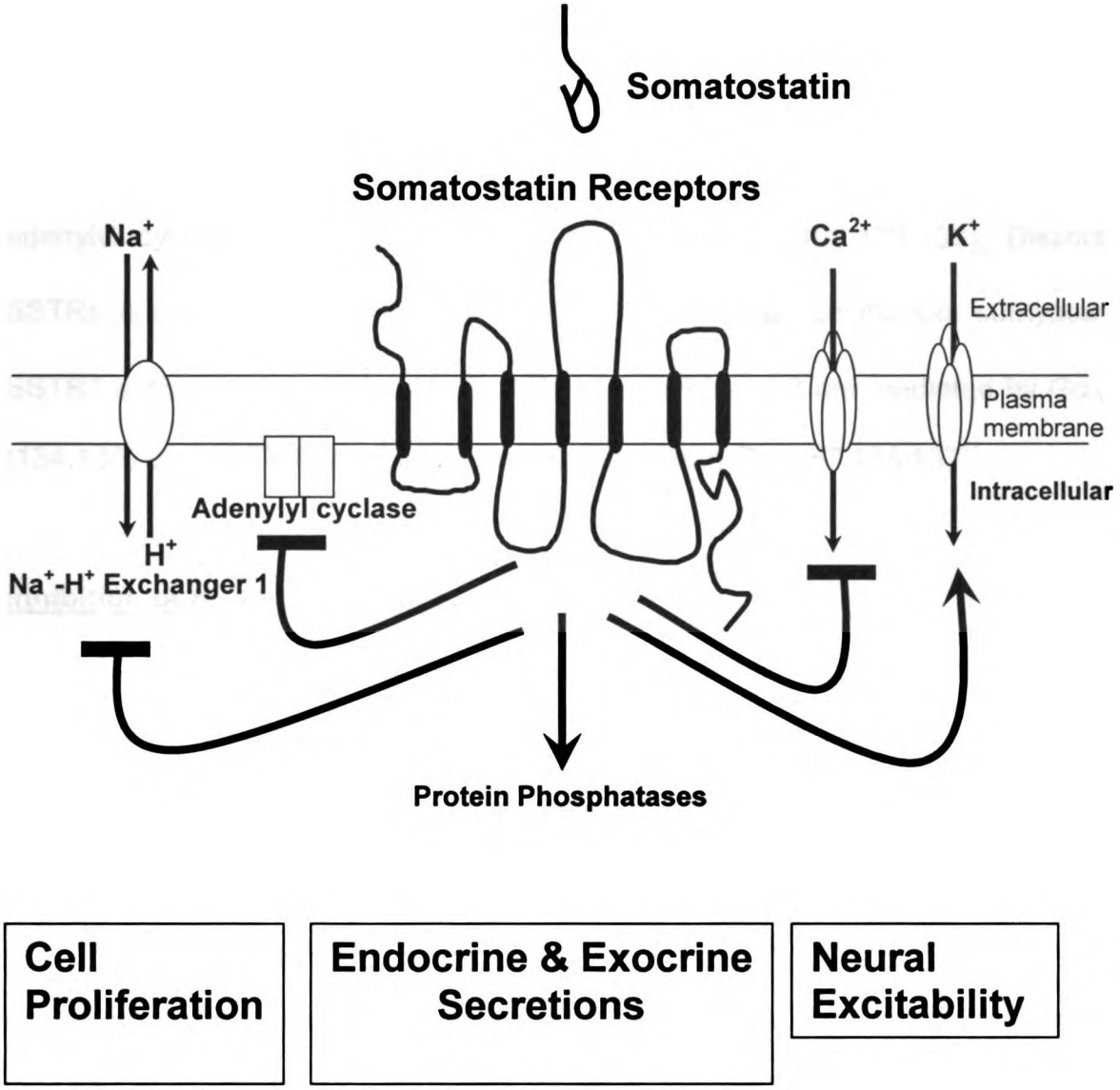

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FIG 1. **Signaling mechanisms used by somatostatin receptors to regulate cell proliferation, endocrine and exocrine secretions, and neural excitability.** Somatostatin activates 5 receptor subtypes, which are coupled to pertussis toxin-sensitive- and insensitive- signaling pathways. Pertussis toxin-sensitive signaling pathways include the inhibition of adenylyl cyclase, the activation of K^+ channels, the inhibition of Ca^{2+} channels, and the activation of protein phosphatases. In contrast, SST acts through a PTX-insensitive pathway to desensitize SSTR and to inhibit the Na^+-H^+ exchanger NHE1.

Inhibition of Adenylyl Cyclase

All five SSTR subtypes inhibit adenylyl cyclase (AC) activity through distinct PTX-sensitive pathways (99,109,129-133). All five SSTR subtypes inhibit adenylyl cyclase (AC) activity with equal efficacy (99,109,129-133). Distinct SSTRs are found to couple to the inhibition of AC via specific $G\alpha_i$ subtypes. SSTR1 actions are mediated by $G\alpha_{i3}$ (131), SSTR2 and 3 are mediated by $G\alpha_{i1}$ (134,135) and SSTR4 and 5 couple through $G\alpha_{i2}$ and $G\alpha_{i3}$ (87,136,137).

Inhibition of K^+ and Ca^{2+} Conductance

SSTRs mediate the modulation of neurotransmission and secretion in part by increasing and decreasing, respectively intracellular K^+ and Ca^{2+} concentrations, which decreases the excitability of neural membranes. SSTRs couple to the activation of several types of K^+ channels, including delayed rectified K^+ channels (115), ATP-sensitive K^+ channels (138), and large conductance Ca^{2+}- and voltage-activated K^+ BK channels (139). Activation of these K^+ channels by SST increases K^+ efflux, leading to hyperpolarization and inhibition of neural transmission and secretion of excitable cells. Activation of SSTRs also decreases intracellular Ca^{2+} by inhibiting the influx of Ca^{2+} through the attenuation of voltage-dependent Ca^{2+} channels (116,117,140) via $G\alpha_o2$ (141). The decreased influx of Ca2+ in turn lowers membrane potential and prevents depolarization of excitable cells.

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Regulation of Mitogenic Pathways through protein phosphatases

All subtypes of SSTR modulate the Erk-dependent mitogen-activated protein kinase (MAPK) cascade. SSTR2, 3, and 5 inhibit Erk activity, although each of these receptor subtypes uses distinct signaling pathways to achieve this effect. In neuroblastoma cells SSTR2 inhibits Erk activity via a protein tyrosine phosphatase-dependent mechanism (142), but in NIH3T3 fibroblasts SSTR3 acts through inactivation of the serine/threonine kinase Raf-1 in NIH3T3 cells (124), and in CHO cells SSTR5 acts through inhibition of guanylate cyclase and cGMP formation (143). Whether these different mechanisms for inhibiting Erk activity reflect distinct receptor subtype-specific properties, or whether they merely reflect cell type-specific differences remains to be determined because Erk inhibition by these three receptors has not been determined in the same cell type. In marked contrast to the inhibition of Erk by SSTR2, 3, and 5, SSTR1 and 4 stimulate activity in CHO-K 1 cells, despite the fact that both receptor subtypes inhibit cell proliferation (136,144). Activation of Erk by SSTR1 requires activation of the protein tyrosine phosphatase SHP-2 and is associated with increased expression of the cyclin-dependent protein kinase inhibitor p21(Cip1/WAF1) and an inhibition of cell proliferation (144). This paradoxical activation of protein tyrosine phosphates SHP-2 and Erk coupled to the inhibition of cell cycle progression is associated only with the SSTR1 subtype.

The regulation of mitogenic pathways by SSTRs is mediated by distinct protein phosphatases. The widely expressed protein tyrosine phosphatase SHP-2 is activated by SSTR1, 2, 3, and 4, but not by SSTR5, (145,146). In contrast,

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SSTR2 is the only SSTR subtype shown to activate the SHP-1 protein tyrosine phosphatase and this is an essential component of SSTR2 inhibition of cell proliferation (147-149).

Induction of Apoptosis and Cell Cycle Arrest

The antiproliferative effects of SSTRs occur through either cytotoxic apoptosis or cytostatic cell cycle arrest. S STR3 is the only subtype shown to induce apotosis (64). The apoptotic effect of SSTR3 is associated with the activation of tumor suppressor protein p53 as well as induction of the pro-apoptotic protein Bax (64,150). Recruitment of SHP-1 to the membrane by SST is an early event required in its antiproliferative signaling that induces intracellular acidification-dependent apoptosis (151). SST-induced apoptosis is associated with activation of a cation insensitive acidic endonuclease and intracellular acidification (152). SSTR3 activates the caspase-8 mediated intracellular acidification, which leads to the mitochondria dysfunction and apoptosis (66).

In contrast, SSTR1, 2, 3, and 5 inhibit proliferation by inducing cell cycle arrest (65). The mechanism shared by each of these subtypes includes a protein tyrosine phosphatse-dependent induction of the tumor suppressor retinoblastoma protein Rb and G1 cell cycle arrest (65). In CHO cells expressing SSTR2, SST induces cell cycle arrest through up-regulation of the cyclin-dependent kinase inhibitor p27(Kip1), which is further maintained by SHP-1 activity (149). Consequently, decreased p27(Kip1)-csk2 association, the inhibition of cyclin E-

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cdk2 kinase activity, and the induction of Rb protein inhibits S-phase entry and G1 cell cycle arrest (149).

Inhibition of Na^+-H^+ Exchanger NHE1

SST also inhibits activity of the ubiquitously expressed Na^+-H^+ exchanger NHE1 (109,126,127). NHE1 is a plasma membrane ion exchanger that catalyzes an electroneutral exchange of extracelluar Na^+ and intracellular H^+ (153). In conjunction with anion exchangers, NHE1 regulates intracellular pH (pH_i) homeostasis and cell volume (154). SST inhibition of NHE1 attenuates H^+ extrusion, resulting in a decrease in intracellular pH_i. Through its regulation of pH_i and cell volume, NHE1 has an established permissive effect in promoting cell proliferation (155-157). Although speculative, the decrease in pH_i associated with SST inhibition of NHE1 could contribute to SST inhibition of cell proliferation and an acidification-induced apoptosis. NHE1 activity is not regulated by changes in cAMP levels, and accordingly its inhibition by SST is independent of cAMP accumulation and is insensitive to PTX (109,126,127). Using heterologously expressed SSTR in fibroblasts, Hou et al found that SSTR1, but not SSTR2, couples to the inhibition of NHE1 (109). Additionally, using chimeric SSTR2/SSTR1 receptors, Hou et al., (109) found that intracellular loops 2 (IL2) and 3 (IL3) of SSTR1 are required to confer SST inhibition of NHE1. These findings indicate that IL2 and IL3 of SSTR1 contain critical amino acids that confer coupling to NHE1 inhibition. The specific amino acid residues necessary to confer coupling to NHE1, however, have not been identified. Moreover,

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whether SSTR subtypes other than SSTR1 regulate NHE1 has not been determined. Chapter 1 of this thesis focuses on SST regulation of NHE1, by determining the coupling of SSTR subtypes and the receptor determinants required.

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Na^+-H^+ EXCHANGER NHE1

Na^+-H^+ exchangers constitute a family of five (NHE1-5) integral plasma membrane ion exchangers catalyzing the electroneutral exchange of extracellular Na^+ for intracellular H^+ with stoichiometry of 1:1 (153). Among the family, NHE1 is the only ubiquitously expressed isoform that functions primarily in pH_i homeostasis and cell volume regulation (154). In contrast, isoforms 2-5 have a more limited tissue distribution and are predominantly expressed in epithelial cells where they function primarily in Na^+ reabsorption. Increases in NHE1 activity and resulting increases in pH_i provide a permissive signal for cell proliferation (156-159), differentiation (160,161), and cell adhesion (162-164). Increases in NHE1 activity are also associated with pathological conditions, such as neoplastic transformation (165,166), tumor invasion (167), and essential hypertension (168,169).

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Functional Importance of Na^+-H^+ Exchanger NHE1

Activation of NHE1 provides a permissive signal for cell proliferation. Growth factors and growth-promoting hormones induce an acute activation of NHE1. Activation of NHE1 is one of the most universal responses to these mitogens. In the absence of mitogens, proliferation can be induced by an exogenously imposed cytoplasmic alkalization, which indicates pH_i as an important component of this response (156). In the presence of mitogens, cell growth is dependent on NHE1, because it is inhibited in NHE1-expressing cells treated with amiloride analogues and in mutant cells devoid of NHE1 (155).

NHE1-deficient cells also have a reduced capacity to grow tumors in immune deficient mice (155).

Increases in NHE1 activity and the resulting increase in pH_i also provide a permissive signal for cell differentiation. Increased NHE1 gene expression is an early event in the differentiation of neuronal and hematopoietic cell lines (100,161,170,171), and elevation of pH_i is observed in the vitamin D3-induced monocytic differentiation and retinoic acid-induced granulocytic differentiation of HL-60 cells (160,172). Although both NHE1 and the Na-HCO_3 co-transporter exist in HL-60 cells as pH_i regulating mechanisms, an increase in only NHE1 activity is noted during the differentiation of these cells (172).

The finding of a spontaneous mouse mutant with slow wave epilepsy has offered insight to the role of NHE1 in development and neural excitability (173). The gene defect in this mouse model has been identified as a null allele of NHE1 with a premature stop codon located between eleventh and twelfth putative transmembrane domains (173). This mutation in NHE1 leads to selective neuronal degeneration in the cerebellum and vestibular apparatus through mechanism other than apoptosis. The loss of NHE1 also leads to neuronal excitability defects. The phenotypic expressions of ataxia and slow-wave epilepsy in slow-wave epilepsy mutant mice suggests an imbalance of ionic homeostasis, specifically by the loss of NHE1.

Recently, a previously unrecognized function of NHE1 as a plasma membrane anchor for the actin-based cytoskeleton was determined (157). Cytoskeletal anchoring by NHE1 occurs independently of ion translocation and is

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mediated by a direct association with the ERM (ezrin, radixin, moesin) family of actin-binding proteins. A FERM domain at the N-terminus of ERM proteins binds directly to the juxtamembrane region of the cytoplasmic domain of NHE1, and an actin-binding domain at the C-terminus of ERM protens binds directly to actin filaments. Through these associations, ERM proteins link actin filaments to the plasma membrane and NHE1 therefore acts as a membrane anchor for polymerized actin. In fibroblasts, NHE1 is not uniformly distributed along the plasma membrane, but is clustered at the leading edge of membrane protrusions, or lamellipodia. This specialized localization of NHE1 is maintained by ERM binding and both functions of NHE1, as a cytoskeletal anchor and as an ion transport protein, play a critical role in cell adhesion, cytoskeleton organization, and cell migration (157,164,174,175).

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Signaling Pathways Regulating Na^+-H^+ Exchanger NHE1 Activity

NHE1 activity is regulated by multiple extracellular signals. Osmotic stress and cytokines stimulate NHE1 activity, as do growth factors acting at receptor tyrosine kinases, hormones and neurotransmitters acting at GPCR, and extracellular matrix proteins acting on integrin receptors (Fig. 2).

Receptor tyrosine kinases activate NHE1 through a Ras-dependent ERK cascade (176,177). Ras activation of NHE1 requires activity of Raf1 (176), the MAP kinase kinase MEK (176), and ERK (177) (Fig. 2). Recently, growth factor activation of NHE1 was found to mediated by the serine-threonine kinase $p90^{rsk}$,

which acts downstream of ERK to directly phosphorylate the cytoplasmic domain of NHE1 and stimulate NHE1 activity (178) (Fig. 2).

GPCR activate NHE1 though either $G\alpha_q$ and $G\alpha_{13}$, because of the 16 identified α subunits of heterotrimeric G proteins, only $G\alpha_q$ and $G\alpha_{13}$ couple to the stimulation of NHE1 (179-181) (Fig. 2). $G\alpha_q$ activates NHE1 through a protein kinase C (PKC)-dependent mechanism (179) (Fig. 2), while $G\alpha_{13}$ activation of NHE1 is mediated by the low molecular weight GTPase RhoA (176) (Fig. 2), and the RhoA kinase ROCK. ROCK is a serine-threonine kinase (174) that directly phosphorylates serine residues at the distal C-terminus of NHE1 (Fig. 2). In contrast to the stimulation of NHE1, some GPCR, including SSTR1 and D2-dopamine receptors, couple to the attenuation of NHE1 activity, (109,126,182). Although the signaling pathways linking GPCR to the attenuation of NHE1 activity remain unknown, recent findings indicate that activation of the G protein $G\alpha_{12}$ inhibits NHE1 (183).

Activation of integrin receptors by extracellular matrix proteins stimulates NHE1 activity (184) (Fig. 2), and recent findings suggest that signals from integrin receptors converge at the level of RhoA with signals from GPCR to activate NHE1 through a ROCK-dependent mechanism (164) (Fig. 2). Moreover, NHE1 is necessary for the fidelity of integrin signaling. In fibroblasts null for NHE1, cell adhesion and spreading is inhibited, the accumulation of integrins, paxillin, and vinculin at focal contacts is impaired, and integrin-regulated tyrosine phosphorylation of p125(FAK) is attenuated (164). Stable expression of NHE1 restores cell adhesion, focal complex assembly, and FAK phorphorylation.

Moreover, NHE1 effects on integrin-dependent cytoskeleton organization are mediated cooperatively through the dual functions of NHE1 as a cytoskeletal anchor (157) and as an ion transport protein (Denker and Barber, manuscript submitted).

Because NHE1 activity provides a critical signal for cytoskeleton organization and cell adhesion, its regulation should be linked to dynamic changes in the cytoskeletal architecture. Consistent with this hypothesis, the Rho-dependent activation of NHE1 by GPCRs for thrombin and lysophosphatidic acid is necessary for associated changes in cytoskeletal dynamics (164,174). Additionally, inhibition of NHE1 activity by pharmacological agents, such as the amiloide analog EIPA, prevents the assembly of actin filaments and focal adhesions in response to thrombin and lysophosphatidic acid (164,175). Whether GPCRs coupling to the inhibition of NHE1 also act to inhibit cytoskeletal reorganization and cell adhesion remains unknown. To date, only GPCR stimulating a Rho-dependent cytoskeletal reorganization have been identified. The focus of Chapter 2 of this thesis is on SSTR1 regulation of RhoA and cytoskeletal organization. The work in this chapter explores the hypothesis that because SSTR1 couples to the inhibition of NHE1, it also inhibits RhoA activity, actin filament assembly and focal contact formation.

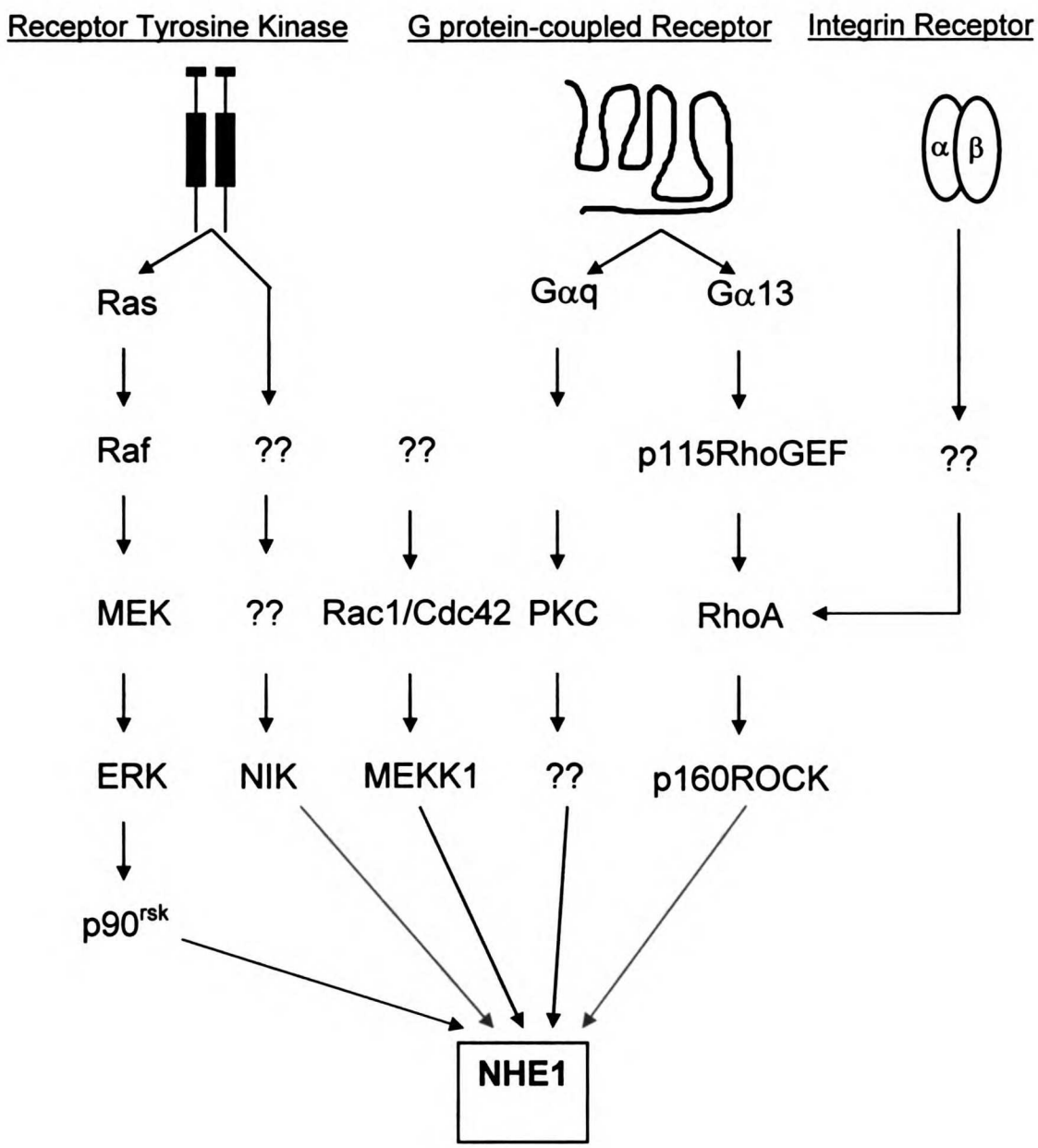

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FIG 2. **GTPase and kinase cascades activate NHE1.** Heptahelical receptors use heterotrimeric G proteins to transduce signals from the receptors to their downstream effectors after activated by extracellular ligands or signals. Of the 16 identified α subunits of heterotrimeric G proteins, only $G\alpha_q$ and $G\alpha_{13}$ couple to the stimulation of NHE1. Receptor tyrosine kinases activate NHE1 through a Ras-dependent ERK kinase cascade.

CHAPTER II

MOLECULAR MOTIFS FOR SUBTYPE-SPECIFIC COUPLING OF SOMATOSTATIN RECEPTORS TO INHIBITION OF THE Na^+-H^+ EXCHANGER NHE1

Members of the superfamily of G protein-coupled receptors (GPCRs) share a heptahelical transmembrane topology and similar mechanisms for transducing signals through trimeric G proteins to intracellular effectors. Subfamilies of GPCRs, distinguished by sequence identity, generally include several receptor isoforms or subtypes that can be activated by similar ligands. Although receptor subtypes within a GPCR subfamily often regulate similar intracellular effectors, they can also signal to distinct effectors and signaling networks through a process termed receptor subtype-specific signaling. Using the subfamily of somatostatin receptors as a model, the objective of this study was to determine a molecular basis for subtype-specific signaling by GPCRs.

The somatostatin receptor family includes five subtypes (SSTR1 to SSTR5) (83,85,86,185,186) that belong to the class A group of GPCRs. Each member is activated by the neuroendocrine peptide somatostatin-14 (SST). SST inhibits diverse cell functions such as hormone secretion, neurotransmitter release, smooth muscle contractility and cell proliferation. These cellular effects are mediated by coupling of the five SSTR subtypes to similar as well as distinct effectors and signaling networks. All five subtypes couple to the inhibition of adenylyl cyclase (133) and the activation of tyrosine phosphatases (65,122-

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125,144,187). Different SSTR subtypes, however, also regulate distinct effectors. SSTR2 to 5, but not SSTR1, activate the GIRK1 inwardly rectifying K^+ channel (188), SSTR1 and 2 inhibit voltage-activated Ca^{2+} channels (189-191), SSTR2 and 5 stimulate phospholipase C activity (123), and only SSTR4 has been shown to stimulate phospholipase A_2 (136).

SST acting at endogenous SSTRs in enteric endocrine cells (126) and hepatic cells (127) also inhibits activity of the ubiquitously expressed Na-H exchanger NHE1. When heterologously expressed in fibroblasts, SSTR1, but not SSTR2, mediates this effect (109). Using chimeric SSTR2/SSTR1 receptors, we previously found that the collective, but not individual, replacement of intracellular domains 2 (IL2) and 3 (IL3) of SSTR2 with those of SSTR1 is sufficient to confer inhibition of NHE1 (109). This suggested that receptor subtype-selective coupling to NHE1 might require an interactive conformation of these two intracellular domains. From previous studies on GPCR regions critical for selective signaling, a general principle has emerged that the relative contribution of different intracellular domains to the selectivity of G protein recognition, and hence effector coupling, varies among different classes of GPCRs (192). Currently, there is no *a priori* certainty that related structural domains have similar functions in different receptors (193).

In the present study we identified selective amino acid motifs in IL2 and IL3 of SSTRs that confer the inhibition of NHE1. We first extended the investigation of SSTR inhibition of NHE1 and found that, when stably expressed in fibroblasts, SSTR3 and 4, but not SSTR5, mediated SST inhibition of NHE1.

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Using a FASTA search of the Swiss-Prot database and aligning amino acid residues of IL2 and IL3 of 16 SSTRs retrieved with PILEUP, we found that consensus motifs of T(S,P)V in IL2 and QQ in IL3 were present in NHE1-coupled SSTR1, 3, and 4, but absent in SSTR2 and 5, which do not signal to NHE1. The collective, but not individual, replacement of cognate amino acids in SSTR2 with these motifs was sufficient to confer SST inhibition of NHE1. Moreover, we found that these IL2 and IL3 motifs are present in selective subtypes of class A GPCRs within the D_2-dopamine, α_2-adrenergic, and thromboxane TxA_2 subfamilies. Together these findings provide a molecular basis for receptor subtype-specific signaling, and they suggest that common motifs shared by distinct subfamilies of GPCRs can be used to regulate a common effector.

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MATERIALS AND METHODS

Materials—Somatostatin-14 was purchased from Bachem (Torrance, CA). Radioactive [^{125}I-Tyr^{11}] SST-14 was obtained from Amersham Corp. The QuikChange Site-Directed Mutagenesis Kit was obtained from Strategene Corp. Reagents for cAMP radioimmunoassay were purchased from Biomedical Technologies (Stoughton, MA). 2,7-Biscarboxyethyl-5(6)-carboxy-fluorescein (BECEF) and nigericin were obtained from Molecular Probes (Eugene, OR). Unless otherwise noted, all other reagents were purchased from Sigma Corp.

Receptor constructs, Cell culture, and Transfection—The cDNA for *r*SSTR3 and *r*SSTR4 were provided by J. Hadcock (Cyanamid Corp). *r*SSTR3 and *r*SSTR4 were subcloned into pcDNA1 (Invitrogen) at EcoRI/XbaI sites. Mouse fibroblast Ltk$^-$ cells, maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS), were transfected with SSTR1-4 using Ca^{2+}-phosphate precipitation as previously described (109). The cells were co-transfected with pRSVneo. G418-resistant clones were selected and SSTR expression was determined by radioligand binding of [^{125}I-Tyr^{11}] SST-14. After three independent transfections, we were unable to obtain Ltk$^-$ cells stably expressing SSTR5. We therefore used CHO-K1 cells stably expressing SSTR3 and SSTR5, provided by Y.C. Patel (Royal Victoria Hospital and Montreal Neurological Institute, Canada). CHO-K1 cells were maintained in F-12 Hamms medium containing 10% FCS and 0.2 mg/ml G418. hSSTR1 and 2 were obtained from G. Bell (University of Chicago)and subcloned into the mammalian

expression vector pCMV at HindIII/XbaI sites. *h*SSTR2 mutants, *h*SSTR2$_{2TV}$, *h*SSTR2$_{3WQQ}$, and *h*SSTR2$_{2TV3WQQ}$, were constructed by replacing arginine-threonine (RT) in IL2 and serine-serine-lysine (SSK) in IL3 of hSSTR2 with cognate threonine-valine (TV) and tryptophan-glutamine-glutamine (WQQ) of *h*SSTR1 respectively or collectively, using the QuikChange Site-Directed Mutagenesis Kit (Strategene, Inc.) with specifically-designed primers of CAAGTGGAGGAGACCCACGGTGGCCAAGATGATCACCATG & CATGGTGATCATCTTGGCCACCGTGGGTCTCCTCCACTTG for IL2 mutation and CTCTGGAATCCGAGTGGGCTGGCAGCAGAGGAAGAAGTCTGAGAAGAAG & CTTCTTCTCAGACTTCTTCCTCTGCTGCCAGCCCACTCGGATTCCAGAG for IL3 mutation. Indicated mutations were confirmed by sequencing. cDNA of *h*SSTR1, *h*SSTR2, and *h*SSTR2 mutants were co-transfected with pRSVneo into clonal CCL39 fibroblasts, which do not express endogenous SSTR, using the Transfast transfection reagent (Promega, Inc.). Clones stably expressing wild-type and mutant SSTR were selected by G418 resistance (0.6 mg/ml) and by their ability to bind [^{125}I-Tyr^{11}]somatostatin-14. CCL39 cells were maintained in DMEM supplemented with 5%FCS.

Radioligand binding assay—Receptor expression, in membranes and intact cells, was determined by measuring binding with [^{125}I-Tyr^{11}] SST-14 (Amersham, Inc.) (109). Radioligand binding to membranes was used to determine receptor expression in the cell clones stably expressing SSTR1-5. Cell membranes,

prepared by hypotonic lysis of cells, were resuspended in 50 mM Tris/HCl (pH7.4) containing 5 mM $MgCl_2$, 0.2 mg/ml bacitracin, 20 μg/ml leupeptin, 1 μg/ml soybean trypsin inhibitor, and 20 μg/ml aprotinin. 10 μg of membrane was incubated in the resuspension buffer containing 0.06 nM of [^{125}I-Tyr^{11}]somatostain-14 (Amersham, Inc.) (specific activity, 2000 Ci/mml) for 60 min at 30 °C, in the absence (total binding) or presence of 100 nM SST-14 (nonspecific binding). The binding reaction was terminated by vacuum filtration over Whatman GF/F glass filters. Filters were washed three times with resuspension buffer and bound radioactivity was counted in a Packard γ spectrometer. Specific binding was determined by subtracting the nonspecific binding from the total binding. Data represent the mean ± standard deviation (S.D.) of three independent membrane preparations. Radioligand binding to intact cells was used to determine the receptor expression of CCL39 cell clones stably expressing *h*SSTR1, *h*SSTR2, and *h*SSTR2 mutants. Cells were plated in 6-well plates and grown for 2 days at 37°C. Cells were then washed with Hanks' balanced salt solution (HBSS) supplemented with 0.1% bovine serum albumin and 2 mM glutamine and incubated for 2 hr at 37°C in this same solution containing [^{125}I-Tyr^{11}]somatostain-14 in the absence (total binding) or presence of 100 nM SST-14 (nonspecific binding). Cells were washed three times with HBSS and lysed in 1n NaOH. Bound radioactivity and specific binding were determined as described as above. Data represent the mean ± S.D. of three independent cell preparations. The half-maximal inhibitory concentration (IC_{50}) of the receptors was determined by incubating cells in HBSS with [^{125}I-

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Tyr[11]]somatostain-14 and serial dilutions of unlabeled SST-14 in the whole-cell radioligand binding assays. Values for IC_{50} were determined from displacement curves using GraphPad Prism 3.0.

Intracellular cAMP Assay—Somatostatin receptor function was confirmed by the ability of SST-14 to inhibit cAMP accumulation. Cells plated at 0.5 x 10^5 cells/well in 24-well plates and maintained for 2 days at 37°C were incubated with 100 μM of the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (IBMX) for 10 min at 37 °C in the absence or presence of 10 μM forskolin, or forskolin plus 100 nM SST-14. Cells were then washed with phosphate-buffered saline and lysed with 5% trichloroacetic acid. The acid-solubilized intracellular cAMP in the cell lysate was determined by using a BTI cyclic AMP Radioimmunoassay kit (Biomedical Technologies, Inc.) according to the suggested protocol of manufacturer. Data represent the mean ± S.D. of three independent cell preparations.

NHE1 Activity—NHE1activity was determined by measuring the rate of pH_i recovery (dpH_i/dt) from an NH_4Cl–induced acid load (109). Cells plated on glass coverslips were serum-starved for 16-20 hr prior to pH_i determinations. Cells were washed and transferred to a nominally $HCO3^-$-free HEPES buffer (194), and loaded with 1 μM of the acetoxymethyl ester of the pH-sensitive fluorescent dye 2,7-Biscarboxyethyl-5(6)-carboxy-fluorescein (BECEF-AM) (Molecular Probe, Inc.) for 10 min at 37°. The coverslips were placed in a cuvette

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maintained in a thermostatically controlled cuvette holder within a Shimazu RF5000 spectrofluometer. BCECF fluorescence was measured at 530 nm by alternately exciting the dye at 500 and 440 nm. The emission ratio was calibrated to pH for each determination by using the high K^+/nigericin (Molecular Probe, Inc.) technique (195). To determine NHE1 activity, cells were pulsed for 10 min with 30 mM NH_4Cl. The rate of pH_i recovery (dpH_i/dt) from an acid load induced by the rapid removal of NH_4Cl was calculated at pH_i intervals of 0.05 units by measuring slope of the pH_i tracing at the indicated pH_i. Changes in quiescent NHE1 activity were determined by adding 10% fetal bovine serum in the absence and presence of 100 nM somatostatin during the NH_4Cl prepulse and HCO^{3-} recovery. Data represent the mean ± S.D. of five to seven measurements.

RESULTS

Expression and function of heterologously expressed SSTR1-5—To determine whether additional SSTR subtypes share the ability of SSTR to inhibit NHE1, we established Ltk$^-$ fibroblast clones stably expressing distinct receptor subtypes. This was successfully achieved for SSTR1-4. After three independent transfections we were unable to obtain Ltk$^-$ fibroblasts stably expressing SSTR5. CHO-K1 cells stably expressing SSTR5, however, have been successfully generated by Patel and colleagues (64). To study SSTR5 coupling to NHE1, therefore, we used CHO-R5 cells obtained from Patel and colleagues and used CHO-K1 cells stably expressing SSTR3 (CHO-R3) as a positive control. Receptor expression was determined by binding of [^{125}I-Tyr^{11}]SST to crude cell membranes (Fig. 3A). Antibodies for the selective receptor subtypes that can be used for immunoblotting or immunostaining are not available, hence Western analysis and immunocytochemistry could not be used to confirm the abundance of receptor expression at the plasma membrane.

The function of heterologously expressed SSTR was assessed by determining their ability to mediate SST inhibition of adenylyl cyclase. Previous findings indicate that although there may be selective regulation of Ca^{2+} and K^+ channels by distinct SSTR subtypes, all five subtypes couple to the inhibition of adenylyl cyclase (133). The diterpene forskolin increased cAMP accumulation in each of the cell clones, and consistent with previous findings, each of the SSTR subtypes effectively mediated SST attenuation of cAMP accumulation (Fig. 3B). The more effective inhibition by SSTR2 compared to SSTR1 is consistent with

previous reports in other cell types (109). Together, these data demonstrate the expression and function of each SSTR subtype stably expressed in either Ltk$^-$ or CHO-K1 cells.

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A

(n=3)	Binding (*f*mol/mg)
pRSVneo	0.3 ± 0.02
Ltk-SSTR1	171.2 ± 10.1
Ltk-SSTR2	655.3 ± 17.9
Ltk-SSTR3	77.1 ± 8.8
Ltk-SSTR4	71.1 ± 8.1
CHO-SSTR3	95.0 ± 6.3
CHO-SSTR5	62.0 ± 7.1

B

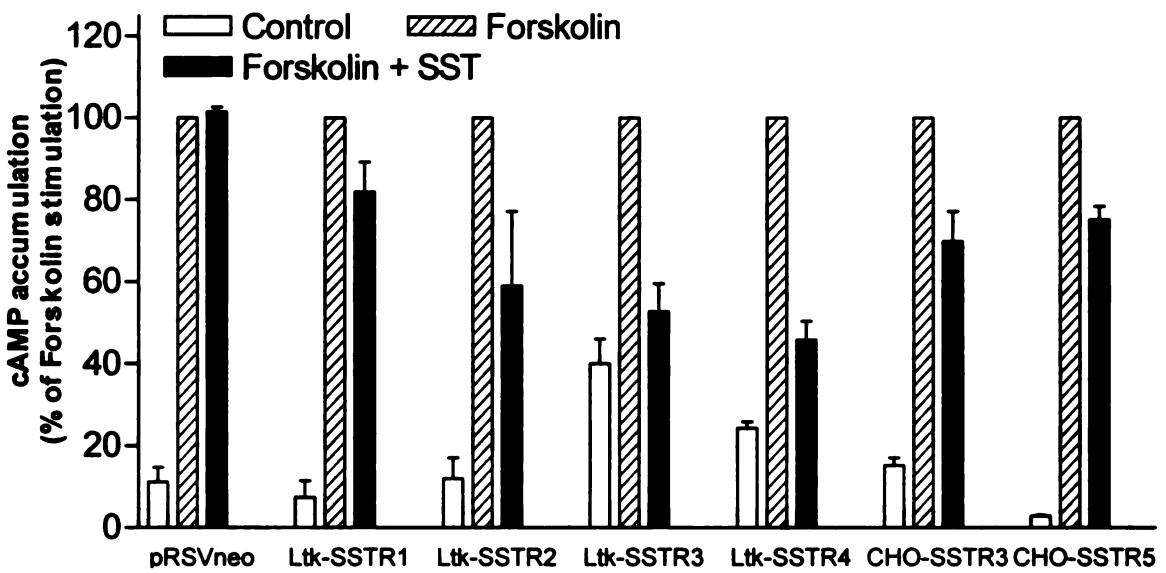

FIG 3. **Functional expression of SSTR1-5.** [^{125}I-Tyr^{11}]somatostatin-14 binding to membranes prepared from cell clones stably expressing SSTR1-5 was used to determine the expressions of SSTR1-5 (A). Specific binding of [^{125}I-Tyr^{11}]somatostatin-14 to the receptors was determined by subtracting the nonspecific binding (in the presence of 100 nM nonradiolabeled somatostatin-14) from the total binding (in the absence of nonradiolabeled somatostatin-14). cAMP accumulation was determined in cells stably expressing SSTR1-5 (B). Determinations were made with no added reagents (*open bars*), with 10μm forskolin in the absence (*hatched bars*) and presence of 100 nM somatostatin (*solid bars*). Data are expressed as a percentage of forskolin-stimulated cAMP accumulation in the absence of somatostatin. Results are presented as the mean ± S.D. of determinations from three experiments.

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Subtype-specific coupling of SSTR1, 3, and 4, but not SSTR2 and 5, to the inhibition of NHE1—Our previous findings indicated that heterologously expressed SSTR1, but not SSTR2, in Ltk^- fibroblasts mediate SST inhibition of NHE1 (109). To determine whether other SSTR subtypes couple to the inhibition of NHE1, we investigated SST effects on activity of NHE1 in cells stably expressing either SSTR3, 4, or 5 and compared this with the response we previously obtained for SSTR1 and 2. SST had no effect on NHE1 activity or pH_i (data not shown). NHE1 activity was determined in a nominally HCO_3^--free HEPES buffer and expressed as the rate of pH_i recovery (dpH_i/dt) from an NH_4Cl-induced acid load. NHE1 activity in the presence of serum was inhibited by SST (100 nM) in cells expressing SSTR1, SSTR3, or SSTR4, but not in cells expressing SSTR2 or SSTR5 (Fig. 4A-F). Because inhibition of NHE1 results in an intracellular acidification, we also confirmed that the steady-state pH_i in the presence of serum was decreased by addition of SST in cells expressing SSTR1, 3 and 4, but not SSTR2 and 5 (Fig. 4G). These findings indicated that SSTR1, 3 and 4, but not SSTR2 and 5, couples to the inhibition of NHE1 and decrease in pH_i.

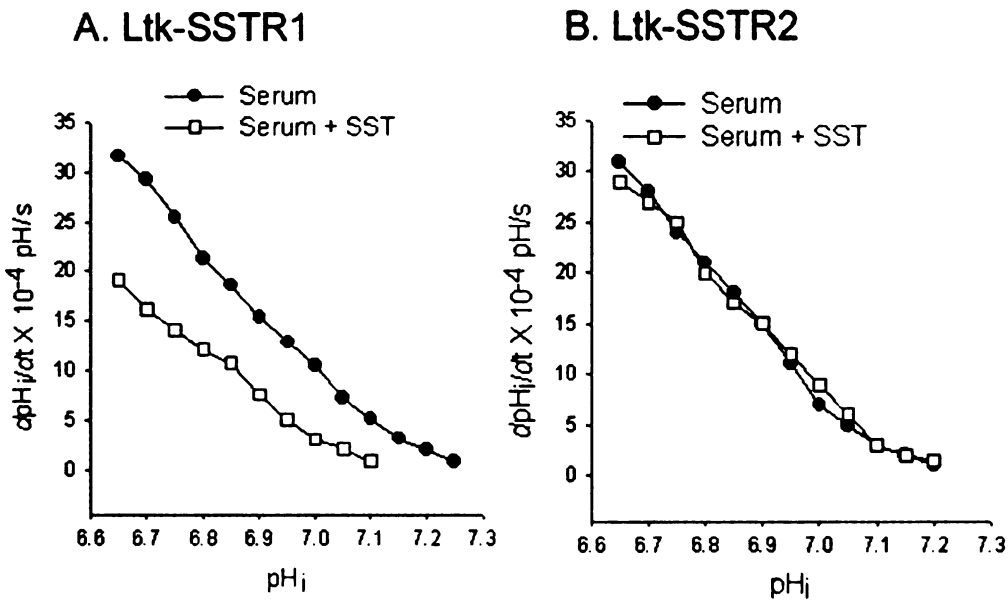
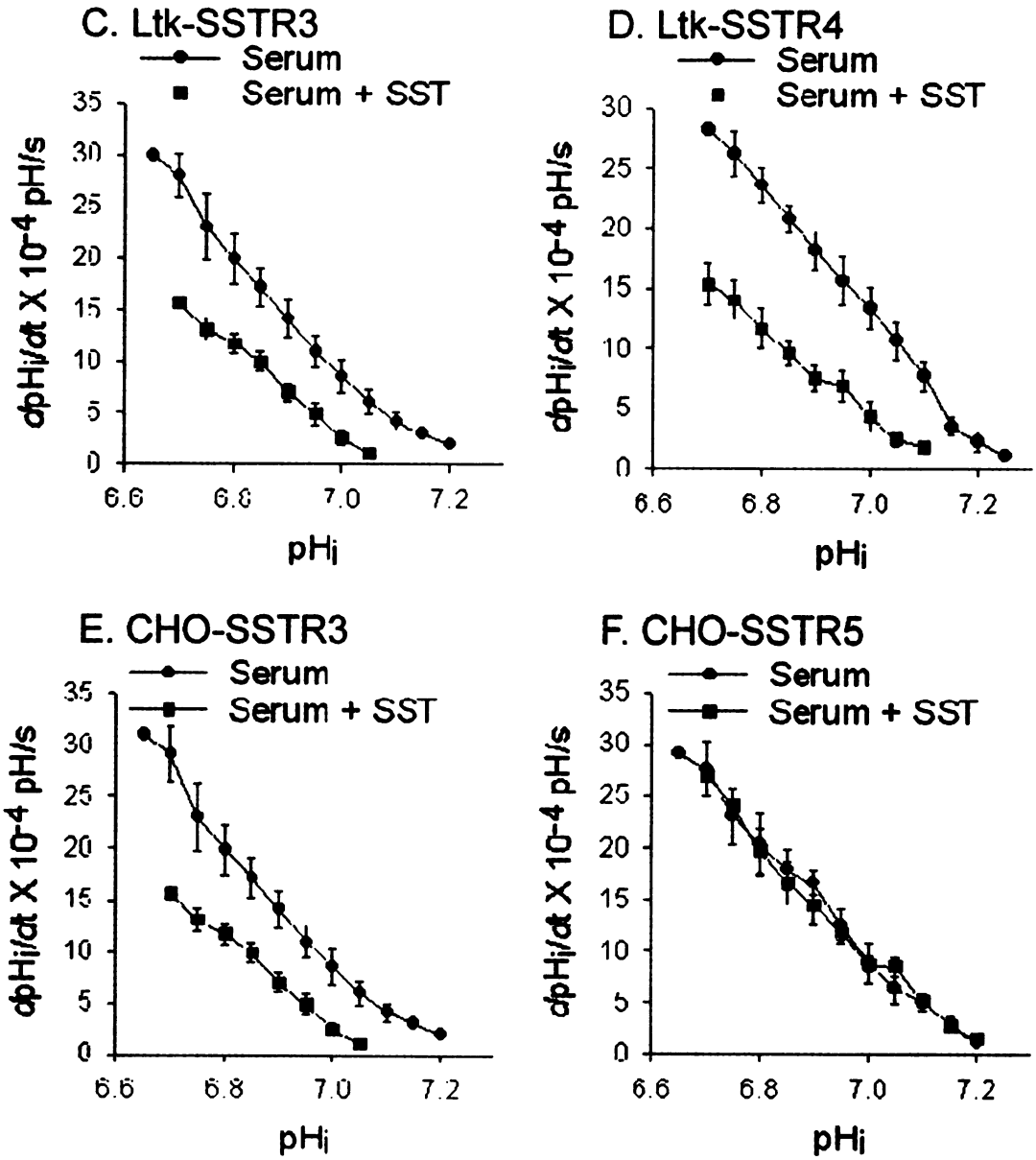


FIG 4. **SSTR1, SSTR3 and SSTR4, but not SSTR2 and SSTR5, inhibit NHE1 activity.** NHE1 activity, expressed as pHi recovery (dpHi/d*t*) from an acid load, was determined in response to 10% serum in the absence and presence of SST. Ltk- cells expressing (A) SSTR1(Ltk-R1), (B) SSTR2 (Ltk-R2), (C) SSTR3(Ltk-R3), or (D) SSTR4 (Ltk-R4). CHO-K1 cells stably expressing (E) SSTR3 (CHO-R3) or (F) SSTR5 (CHO-R5). (G) Serum-induced steady-state pHi of cells expressing SSTR3, 4 and 5 in the absence or presence of 100 nM SST. Results represent the means ± S.D. from four to six cell preparations. Fig. 4C-G are included in the following two pages.



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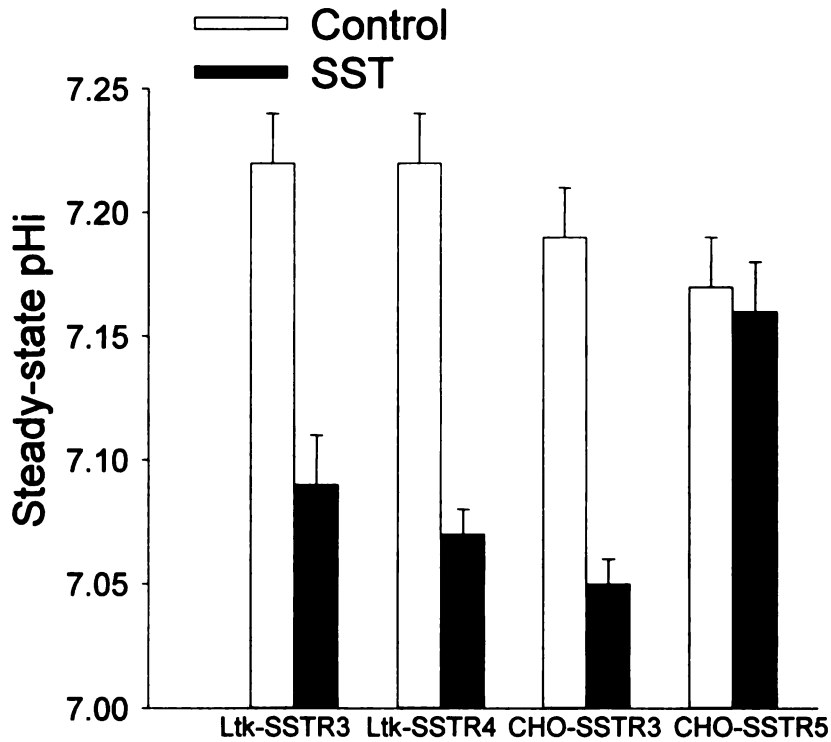
G. Steady-state pHi



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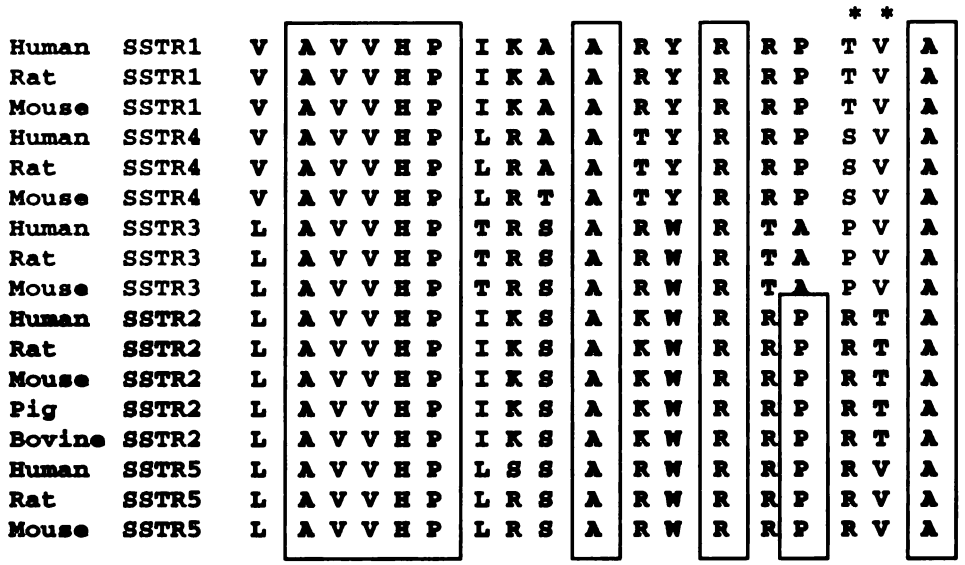
Amino acid sequence alignment reveals conserved subtype-specific residues in intracellular loop 2 and 3 of somatostatin receptors—Using chimeric SSTR2/SSTR1 receptors, we previously determined that both IL2 and IL3 of SSTR1 are required to confer coupling of chimeric SSTR2/1 receptor to the inhibition of NHE1 (109). We therefore sought to determine whether conserved sequences in IL2 and IL3 were shared by SSTR1, 3 and 4, which inhibit NHE1, but not by uncoupled subtypes SSTR2 and 5. The alignment of amino acid sequences in IL2 and IL3 of all known SSTRs showed that IL2 and IL3 are all composed of segments of charged residues bounded by large hydrophobic stretches (Fig. 5). This alignment revealed several amino acid residues that were common in SSTR1, 3, and 4 and others unique to SSTR2 and 5. In IL2, all species of SSTR2 and 5 have a charged arginine residue close to the fourth transmembrane domain, which is absent in SSTR1, 3 and 4. In the middle IL3 segment, SSTR1, 3, and 4 have one or more polar glutamine residues, which are absent in SSTR2 and 5. By comparing these potential residues and their neighboring residues between and within each SSTR subtypes, the amino acid sequence alignment revealed consensus motifs of T(S,P)V in IL2 and (W)QQ(R) in IL3 that were unique to NHE1-coupled SSTR1,3, and 4, but not found in SSTR2 and 5 (Fig. 5). Homology modeling of SSTR1 based on the recently determined structure of rhodopsin (196) suggests that the TV motif is at the C-terminal end of IL2 near the cytoplasmic tip of transmembrane (TM) IV, while QQ is squarely within IL3 (Fig. 6). To determine whether these putative motifs were sufficient to confer coupling to the inhibition of

NHE1, we made site-directed substitutions in *h*SSTR2 by replacing cognate arginine-threonine (RT) residues in IL2 and serine-serine-lysine (SSK) in IL3 of *h*SSTR2 by threonine-valine (TV) in IL2 and tryptophan-glutamine-glutamine (WQQ) in IL3, based on the *h*SSTR1 sequence. These substitutions were made individually in $hSSTR2_{2TV}$ and $hSSTR2_{3WQQ}$ mutants and collectively in the $hSSTR2_{2TV3WQQ}$ mutant.

Intracellular loop 2



Intracellular loop 3

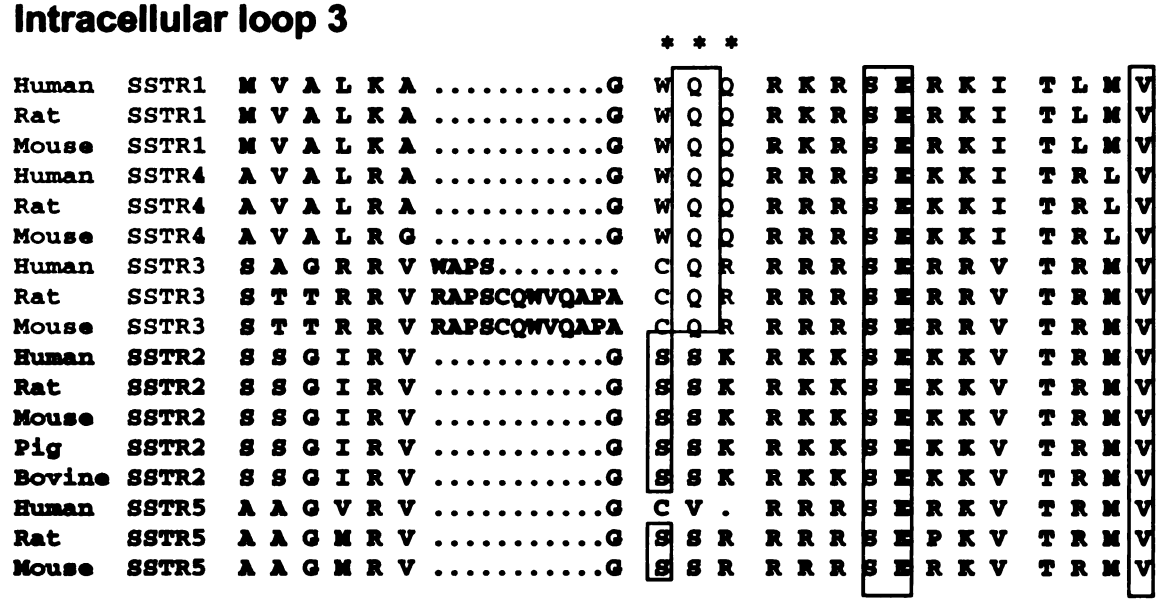


FIG 5. **Alignment of amino acid sequences in the intracellular loop 2 and 3 of all known somatostatin receptors.** Asterisks (*) indicate the positions of he putative motifs for NHE1 coupling.

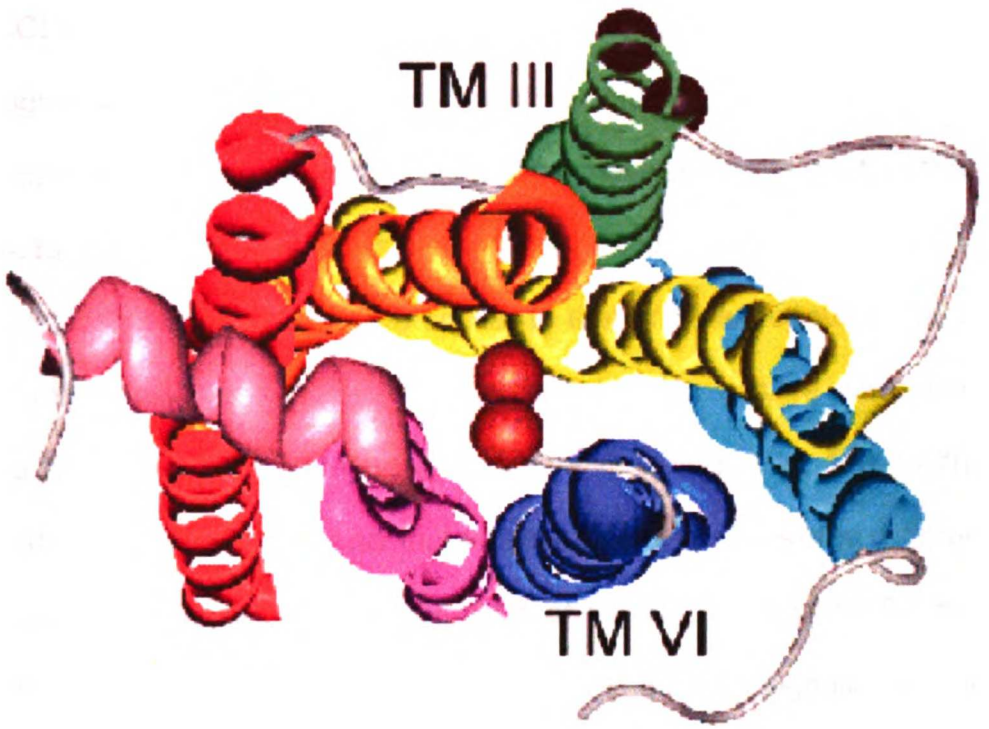


FIG 6. **Intracellular end view from the cytoplasm of the predicted structure of SSTR1 based on the crystal structure of bovine rhodopsin.** The transmemebrane (TM) helices are rainbow color-coded: I. red; II, orange; III, yellow; IV, green; V, light blue; VI, dark blue; and VII, magenta. Helix VIII (non-transmembrane) is pink, and termini and loops are gray. The gap in IL3 represents missing segments 236-239, and the extreme C-terminal tail (334-348) is omitted for clarity. Black spheres represent the positions of IL2-TV, and red spheres represent the positions of IL3-QQ.

Receptor expressions of *h*SSTR1, *h*SSTR2, and *h*SSTR2 mutants in CCL39 cells—Wild type *h*SSTR1, *h*SSTR2, and *h*SSTR2 mutants were stably expressed in CCL39 fibroblasts, which express only the NHE isoform NHE1 (197). CCL39 fibroblasts, rather than Ltk^- fibroblasts, were used because they yielded higher levels of SSTR2 mutant receptors. Radioligand saturation binding in whole cells was used to determine receptor expression and to confirm that wild type CCL39 cells do not express somatostatin-binding sites (Fig. 7A). The expression levels we observed were similar to that of heterologously expressed SSTR in other cell types (198). Competition binding assays were used to determine IC_{50} values for wild-type and mutant receptors (Fig. 7A). Although receptor affinities of wild-type and mutant receptors showed some differences, the high expression levels and high affinities of SSTR1, 2, and SSTR2 mutants exclude the possibilities that the inability of SSTR to mediate signaling due to the impotence of ligand binding. Very interestingly, the lower IC_{50} of SSTR mutants, compared to that of SSTR2, suggested an increase in affinity of SSTR mutants. This increase in affinity of SSTR2 mutants suggested that the mutations might alter receptor conformation or G protein coupling.

***h*SSTR1, *h*SSTR2, and *h*SSTR2 mutants inhibit adenylyl cyclase**—To confirm that SSTR1, SSTR2, and SSTR2 mutants expressed in CCL39 cells were functional, we determined whether they mediated SST inhibition of forskolin-stimulated cAMP accumulation. Consistent with reports that all SSTR subtypes couple to the inhibition of adenylyl cyclase (133), SST inhibited forskolin-

stimulated cAMP accumulation in CCL39 cells stably expressing *h*SSTR1, *h*SSTR2, and *h*SSTR2 mutants (Fig. 7B). In contrast, SST-14 did not inhibit the forskolin-induced cAMP accumulation in control CCL39 cells transfected with pRSVneo only. These findings indicated that mutations in SSTR2 did not affect the coupling of SSTR2 mutants to the inhibition of adenylyl cyclase. SSTR2 mutants still maintained the ability to inhibit adenylyl cyclase, which is a characteristic of all five SSTR subtypes. The relatively greater attenuation of cAMP by SSTR2, compared to SSTR1, has previously been observed (109).

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A

(n=3)	Binding (*f*mol/mg)	IC_{50} (nM)
pRSVneo	1.8 ± 1.5	n/a
*h*SSTR1	546.1 ± 55.4	3.39
*h*SSTR2	88.3 ± 6.4	7.65
*h*SSTR2$_{2TV}$	37.3 ± 8.4	2.88
*h*SSTR2$_{3WQQ}$	36.3 ± 0.6	3.31
*h*SSTR2$_{2TV3WQQ}$	36.7 ± 1.0	1.32

B

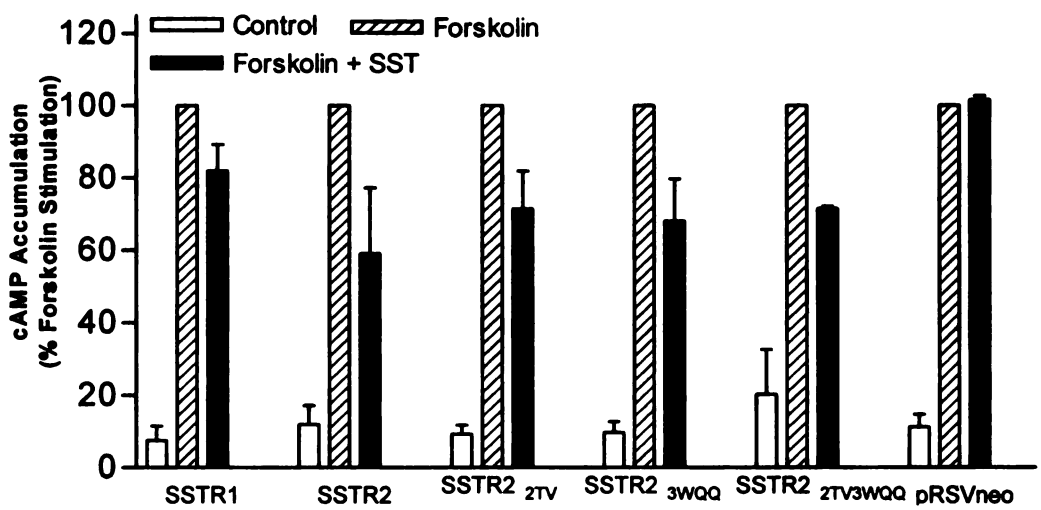

FIG 7. **Functional expressions of *h*SSTR1, *h*SSTR2, and *h*SSTR2 mutants on CCL39 cells.** [^{125}I-Tyr^{11}]somatostatin-14 binding to intact cells stably expressing *h*SSTR1, *h*SSTR2, *h*SSTR2$_{2TV}$, hSSTR2$_{3WQQ}$, and *h*SSTR2$_{2TV3WQQ}$ was used to determine the expressions of these receptors (A). Specific binding of [^{125}I-Tyr^{11}]somatostatin-14 to the receptors was determined by subtracting the nonspecific binding (in the presence of 100 nM nonradiolabeled somatostatin-14) from the total binding (in the absence of nonradiolabeled somatostatin-14). Values for IC_{50} were determined from displacement curves (in the presence of serial dilutions of nonradiolabeled somatostatin-14) using GraphPad Prism 3.0. cAMP accumulation was determined in cells stably expressing *h*SSTR1, *h*SSTR2, *h*SSTR2$_{2TV}$, hSSTR2$_{3WQQ}$, and *h*SSTR2$_{2TV3WQQ}$ (B). Determinations were made with no added reagents (*open bars*), with 10μm forskolin in the absence (*hatched bars*) and presence of 100 nM somatostatin (*solid bars*). Data are expressed as a percentage of forskolin-stimulated cAMP accumulation in the absence of somatostatin. Results are presented as the mean ± S.D. of determinations from three experiments.

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hSSTR2$_{2TV3WQQ}$, but not hSSTR2, hSSTR2$_{2TV}$ and hSSTR2$_{3WQQ}$, couples to the inhibition of NHE1—To determine whether the IL2 and IL3 motifs substituted into SSTR2 conferred inhibition of NHE1, we investigated SST effects on the activity of NHE1 in CCL39 cells stably expressing either SSTR1, SSTR2, or SSTR2 mutants. In cells expressing either SSTR1, SSTR2, or SSTR2 mutants, the acute addition of serum to quiescent cells increased NHE activity (Fig. 8A-E). SST attenuated the serum-stimulated NHE1 activity in CCL39 cells expressing either hSSTR1 (Fig. 8A) or hSSTR2$_{2TV3WQQ}$ (Fig. 8E). In contrast, SST had no effect on the serum-stimulated NHE1 activity in CCL39 cells expressing either hSSTR2 (Fig. 8B), hSSTR2$_{2TV}$ (Fig. 8C), or hSSTR2$_{3WQQ}$ (Fig. 8D). Additionally, serum increased steady-state pH_i, compared to quiescent control pH_i, and this was attenuated by SST in cells expressing SSTR1, 3, or 4, but not SSTR2 or SSTR5 (Fig. 8E). These findings indicate that double substitutions of cognate amino acids in IL2 and IL3 of hSSTR2 with TV and WQQ of hSSTR1, but not single substitutions in these domains, conferred the ability to inhibit NHE1.

To confirm that our findings were not due to clonal variations in SSTR transfected cells, we investigated additional CCL39 clonal cells stably expressing either hSSTR2$_{2TV}$ (32.8 ± 2.0 fmol/mg protein, n=3), hSSTR2$_{3WQQ}$ (13.9 ± 1.5 fmol/mg protein, n=3), or hSSTR2$_{2TV3WQQ}$ (5.4 ± 0.5 fmol/mg protein, n=3). Consistent with the above findings, these additional clones confirmed that SST inhibited serum-stimulated NHE1 activity only in CCL39 cells expressing hSSTR2$_{2TV3WQQ}$ (Fig. 9). In CCL39 cells expressing either hSSTR2$_{2TV}$ or hSSTR2$_{3WQQ}$, somatostatin did not inhibit the serum-stimulated NHE1 activity.

These findings suggested that the coupling of *h*SSTR2$_{2TV3WQQ}$, but not *h*SSTR2$_{2TV}$ and *h*SSTR2$_{3WQQ}$, to the inhibition of NHE1 is not due to clonal variations.

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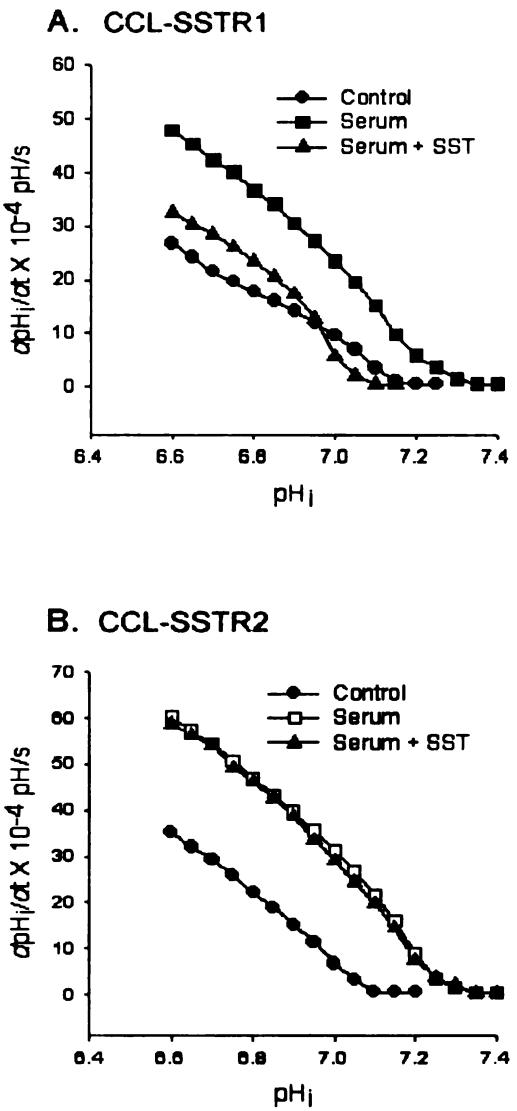
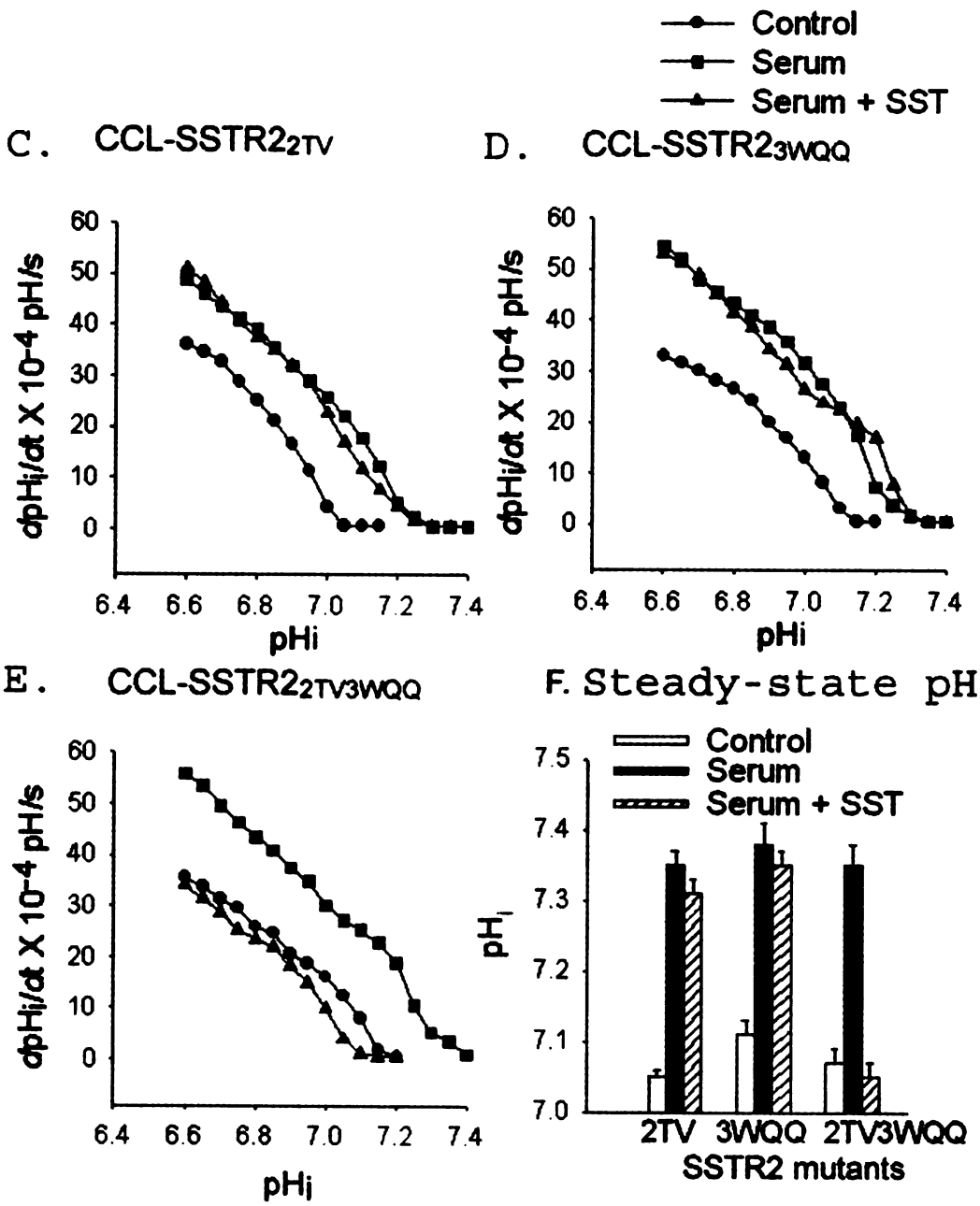


FIG 8. ***h*SSTR1 and *h*SSTR2$_{2TV3WQQ}$ inhibit NHE1 activity.** NHE1 activity was determined in the absence (control) and presence of 10% fetal bovine serum (serum) or 10% fetal bovine serum plus 100 nM somatostatin (serum + SST). as described in the legend to Fig. 4. (A and B) CCL39 cells stably expressing wild-type *h*SSTR1 (CCL-R1)(A) and *h*SSTR2 (CCL-R2). CCL39 cells expressing the SSTR2 mutants (C), *h*SSTR2$_{2TV}$ (CCL-R2$_{2TV}$), (D) *h*SSTR2$_{3WQQ}$ (CCL-R2$_{3WQQ}$), and (E),or hSSTR2$_{2TV3WQQ}$ (CCL-R2$_{2TV3WQQ}$). (F) Steady-state pHi in CCL39 cells expressing the indicated SSTR2 mutants was determined in the absence of serum (control) and in the presence of serum or serum plus somatostatin. Results represent the means ± S.D. from five to seven cell preparations. The following page contains Fig. 8C-F.


Control
Serum
Serum + SST
C. CCL-SSTR2$_{2TV}$
D. CCL-SSTR2$_{3WQQ}$
dpHi/dt X 10^{-4} pH/s
pHi
E. CCL-SSTR2$_{2TV3WQQ}$
F. Steady-state pHi
pH$_i$
2TV
3WQQ
2TV3WQQ
SSTR2 mutants

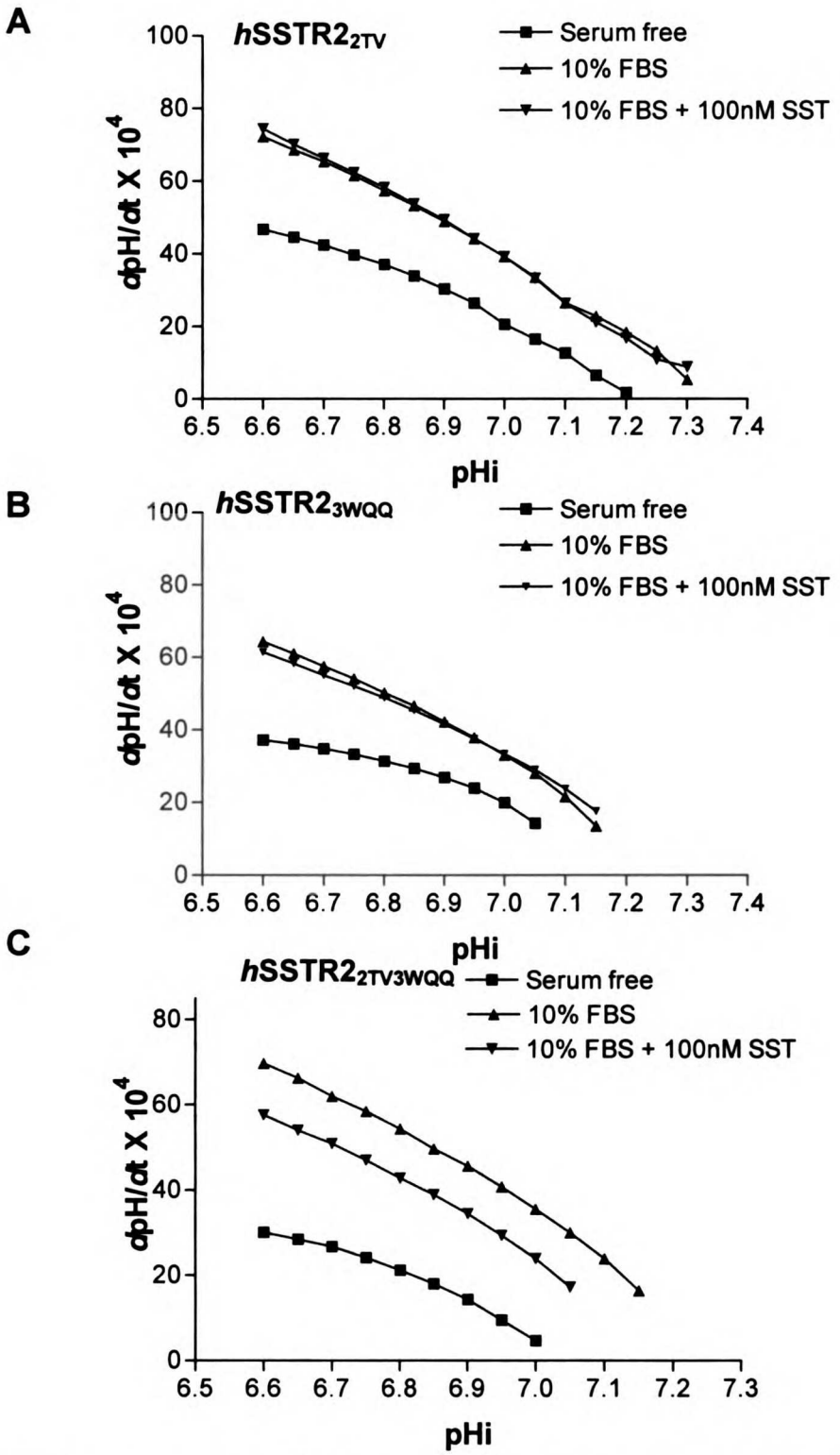


FIG 9. ***h*SSTR2$_{2TV3WQQ}$ in a different CCL39 clone also inhibits serum-stimulated pH$_i$ recoveries (dpH$_i$/d*t*) from an acid load.** In different CCL39 cell clones stably expressing either *h*SSTR2$_{2TV}$ (A), *h*SSTR2$_{3WQQ}$ (B), or hSSTR2$_{2TV3WQQ}$ (C), dpH$_i$/d*t* following a 20 mM NH_4Cl prepulse was determined in the absence and presence of 10% fetal bovine serum or 10% fetal bovine serum plus 100 nM somatostatin. Results represent the means ± S.D. from three experiments.

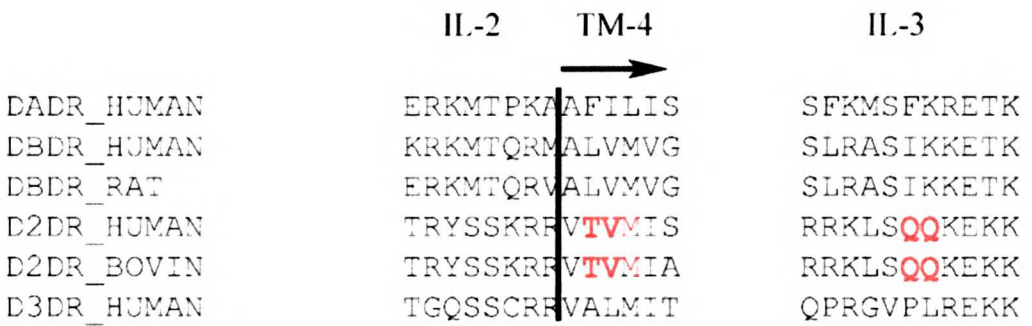
Shared consensus motifs for inhibition of NHE1—Our findings suggest that the amino acid motifs, TV in IL2 and WQQ in IL3 act in concert to provide the molecular basis for coupling SSTR to NHE1 inhibition. Because TV in IL2 and WQQ in IL3 act in concert to provide a molecular basis for SSTR inhibition of NHE1 activity, we investigated whether these motifs occur in other class A GPCRs. We previously determined that endogenously expressed D_2-dopamine receptors in GH3 cells (182) and heterologously expressed D_2-dopamine receptors in Ltk^- cells (199) couple to the inhibition of NHE1. Using the tGRAP alignment database (200), we found that both motifs are present in the D_2-dopamine receptor but not in the D_1- or D_3-dopamine receptors (Fig. 10A). We therefore investigated whether amino acid motifs T(S,P)V near or within IL2 and QQ near or within IL3 were found in other class A GPCRs. SSTR and/or D_2-dopamine family sequences were added when analyzing each family to help identify the exact location of the respective motifs. Alignment of loop regions and their immediate TM extensions using CLUSTALW failed to align either the T(S,P)V or QQ motifs between families. Alignment of IL3 was more difficult than that of IL2 because of large insertions in some families, which made it impossible to use an alignment tool. These loops were therefore aligned manually. There were many instances of these motifs occurring independently, especially the T(S,P)V motif, which is present in all classes of α -adrenergic and C-C type chemokine receptors (data not shown). Co-occurrence of TV in IL2 and QQ in IL3, however, was rare and was found selectively in α_{2B}-adrenergic and thromboxane A_2 receptors (Fig. 10B), although there was no detectable pattern in

the distances of these motifs from TM helices in these receptors compared with SSTR. Although a TV motif is present in α_{2A}- and α_{2B}-adrenergic receptors, the QQ motif in the 2B subtype is not found in the 2A subtype (Fig. 10A). Moreover, as in SSTR1, the co-presence of an IL2-TV and an IL3-QQ in D_2-dopamine, α_{2B}-adrenergic, and thromboxane A_2 receptors is nearly invariant across species (not shown). These data suggest that the presence of T(S,P)V near the TM IV–IL2 transition and the presence of QQ in IL3 might represent conserved motifs that collectively confer coupling to the inhibition of NHE1.

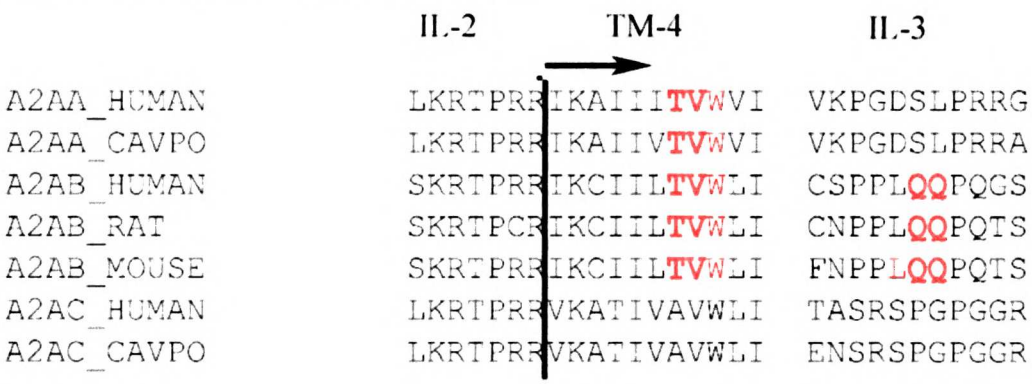
Added Note: Subsequent to the completion of my dissertation work, Anita Joubel in the Barber lab found that when stably expressed in HEK293 cells, the α_{2B}-adrenergic, but not the α_{2A}-adrenergic receptor couples to the inhibition of NHE1.

A.

DOPAMINE RECEPTORS



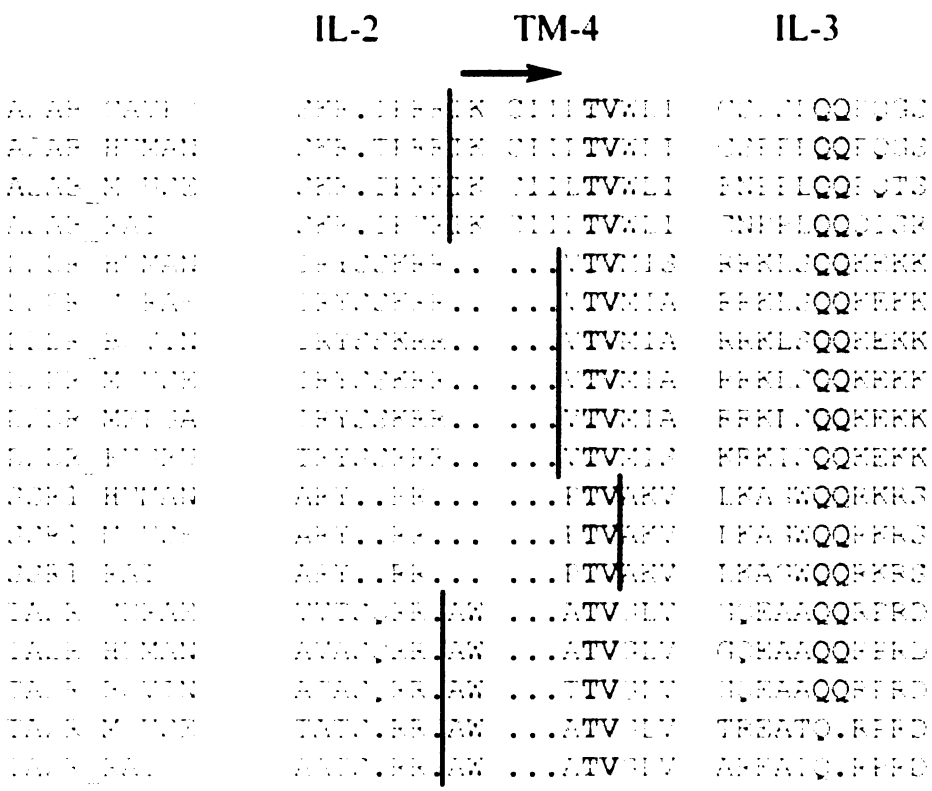
ALPHA ADRENERGIC RECEPTORS



THROMBOXANES

FIG 10. **Co-occurrence of TV and QQ motifs is uniquely conserved in intracellular IL2 and IL3 of α_{2B}-adrenergic, D_2-dopamine, SSTR1, and thromboxane A_2 receptors.** (A) Alignment of IL2 and IL3 in receptor families of dopamine, α_2-adrenergic, and thromboxane A_2 receptors reveals that only select subtypes within each family have both motifs (red). (B) A global alignment of IL2 and IL3 in α_{2B}-adrenergic, D_2-dopamine, SSTR1, and thromboxane A_2 receptors shows that the motifs (red) are invariant in most species (shown in the following page).

B.



DISCUSSION

Previous structure-function studies on GPCRs suggest that there is no *a priori* certainty that related structural domains have similar functions in different receptors (193). We have now identified patterns of amino acid residues that are shared by different subfamilies, but not necessarily by similar subtypes within a subfamily, that may confer coupling to a common effector. Selective residues near the TM IV–IL2 transition and in IL3 of three SSTR subtypes—SSTR1, 3, and 4—that signal to the inhibition of NHE1 are not present in SSTR2 and 5 subtypes, which are not coupled to NHE1 regulation. The collective substitution of these residues in SSTR2, however, was sufficient to switch signaling to the inhibition of NHE1. Moreover, we found that these IL2 and IL3 residues are present in the D_2-dopamine, which we previously confirmed couples to the inhibition of NHE1 (182), and in α_{2B}-adrenergic and thromboxane A_2 receptors.

Studies using site-directed mutagenesis, chimeric receptors, and synthetic peptides have identified residues within the IL2 (201-203) and IL3 (204-207) regions that are critical for G protein and effector coupling. Although selective regions of IL2 and IL3 are essential for G protein recognition, the cooperative interaction of these domains has also been shown to be critical for conferring coupling specificity (208-211). Our finding that the combined, but not individual, replacement of IL2 and IL3 motifs is required to switch SSTR2 coupling to NHE1 supports the requirement for a cooperative interaction between these two domains.

Which hydrophobic domains constitute the molecular basis for subtype-specific signaling, however, remains controversial, and findings suggest critical independent roles for IL2, IL3, and the C-terminal cytoplasmic tail. It is possible that different classes of receptors require distinctly different domains to regulate specific downstream effectors. Previous findings suggest that subtype-specific coupling of SSTR to NHE1 is conferred by the structural domains in both IL2 and IL3 (109). Amino acid sequence alignment of current study revealed consensus motifs of T(S,P)V in IL2 and (W)QQ(R) in IL3 that were unique to NHE1-coupled SSTR1,3, and 4, but not found in SSTR2 and 5. The functions of these putative NHE1-regulatory motifs were confirmed by the NHE1-inhibitory function of *h*SSTR2$_{2TV3WQQ,}$ in which RT in IL2 and SSK in IL3 of *h*SSTR2 were replaced by TV in IL2 and WQQ in IL3 of *h*SSTR1 respectively. The inability of *h*SSTR2$_{2TV}$ and *h*SSTR2$_{3WQQ}$ to inhibit NHE1 suggested that TV in IL2 and WQQ in IL3 act in concert for coupling SSTR to NHE1. Because the expression levels and IC_{50} are similar in CCL39 cells expressing either *h*SSTR2, *h*SSTR2$_{2TV}$, *h*SSTR2$_{3WQQ}$, and *h*SSTR2$_{2TV3WQQ}$, the difference in SST effect on NHE1 activity are likely not due to differences in receptor affinities. The finding that motifs in IL2 and IL3 that confer coupling to NHE1 inhibition by SSTR are also conserved in NHE1-inhibitory D2-dopamine receptor indicated that specific amino acid motifs in IL2 and IL3 of GPCRs confer coupling of receptors to NHE1 inhibition.

Conservation of sequences within the seven TM bundle of GPCR suggests that the three-dimensional structure recently identified for rhodopsin (196) might be shared by most GPCRs. Residues within IL2 and IL3, however, are not highly

conserved, which suggests that intracellular surfaces may be structurally distinct. Recent findings by Chung et al. (212) on the NMR structure of the IL2 region of the α_{2A}-adrenergic receptor indicate that it is predominantly helical, possibly extending part of the TM IV helix, in contrast to its L-shaped structure in rhodopsin (196). In SSTR1 the TV is in IL2 connecting TM III and IV, and QQ is adjacent to TM VI (Figure 3B); the current activation model of GPCR suggests that conformational changes include a separation of TM III and TM VI (213). As viewed from the cytoplasmic face, a change in the orientation of these two TM domains is predicted to occur by a clockwise rotation of TM VI relative to TM III. Moreover, signaling is impaired by mutations that conformationally constrain TM III and TM VI of rhodopsin, either by engineered disulfide bridges (214,215) or by Zn^{2+}-activated metal ion-binding sites (216). If this is correct, a plausible model is that receptor activation would have to change the relative orientation and accessibility from the cytoplasm of both TV, connected to TM III by the IL2 loop, and QQ, connected to TM VI through the IL3 loop. In this scenario, the variable location of TV either on the loop side or on the TM IV side is consistent with penetration of the C terminus of Gα subunits into the membrane to allow for direct interaction with the TM residues.

Divergent signaling by a GPCR to distinct signaling networks can occur at the receptor–G protein interface or at the G protein–effector interface. Divergent signaling by SSTR1 occurs by both mechanisms. SSTR inhibition of adenylyl cyclase and activation of tyrosine phosphatases involve divergent signaling at the G protein–effector interface because both actions are mediated by pertussis

toxin-sensitive $G\alpha_i$ subunits. Divergent signaling by SSTR1, 3, and 4 to adenylyl cyclase and NHE1, however, must occur at the receptor–G protein interface because inhibition of adenylyl cyclase, but not NHE1, is blocked by pertussis toxin (109,126). Distinct receptor contact sites for activating different G proteins have been identified in juxtamembrane regions of IL2 and IL3 of α-adrenergic (217,218) and vasopressin (203) receptors. This is analogous to our findings that these regions selectively confer a $G\alpha_i$-independent inhibition of NHE1. Although the identity of the trimeric G protein coupling SSTR to NHE1 remains to be determined, it is unlikely to be a member of the $G\alpha_s$, $G\alpha_i$, or $G\alpha_q$ families. Expression of mutationally active $G\alpha_s$ and $G\alpha_i$ subunits has no effect on NHE1 activity (180), and activation of $G\alpha_q$, like the effect of $G\alpha_{13}$, stimulates NHE1 (179,180). The only Gα subunit shown to inhibit NHE1 is $G\alpha_{12}$ (183). Expression of mutationally active $G\alpha_{12}$ inhibits only stimulated NHE1 and has no effect on quiescent activity, as we found with SSTR subtypes. If $G\alpha_{12}$ does couple SSTR to the inhibition of NHE1, it might act by inhibiting signaling by $G\alpha_{13}$ or $G\alpha_q$, possibly by sequestering a common guanine nucleotide exchange factor (GEF). Stimulation of NHE1 activity by $G\alpha_{13}$ is mediated by the low molecular weight GTPase Rho (164,176), and $G\alpha_{13}$ activates Rho by stimulating p115RhoGEF (219). Although p115RhoGEF binds to both $G\alpha_{12}$ and $G\alpha_{13}$, its activation of Rho is stimulated by $G\alpha_{13}$, but not $G\alpha_{12}$, and activation of $G\alpha_{12}$ inhibits $G\alpha_{13}$ stimulation of p115RhoGEF (219). Hence, one possible mechanism whereby activated NHE1 is inhibited might be through $G\alpha_{12}$ blocking the $G\alpha_{13}$-

p115RhoGEF-Rho signal. Consistent with this possibility is the finding that thromboxane TxA_2 receptors, which contain the IL2 and IL3 motifs coupling to the inhibition of NHE1, activate $G\alpha_{12}$ (220).

Most GPCR subtypes that are activated by similar ligands share similar sequences and are predicted to have evolved by duplications of a common ancestral gene. We found, however, that the conserved T(S,P)V and QQ motifs are shared by distinct GPCR subfamilies, but not by all subtypes within a given subfamily. This suggests that these subtypes diverged from a common ancestor that coupled to NHE1 through the T(S,P)V and QQ motifs and that these motifs were mostly lost in subsequent divergences that gave rise to families. Alternatively, the appearance of the same motif in some members of different subfamilies may represent convergent evolution to a motif that is linked to NHE1 inhibition. There are not many examples of convergent evolution to a common functional architecture and activity, even in proteins with entirely different structures (221). If the shared "NHE1 motifs" represent convergent evolution, would it necessarily indicate the regulation of a common NHE1-dependent cell function by different GPCR subclasses? Inhibition of cell proliferation is an action shared by decreased NHE1 activity (156,157), SSTR1 (222,223), and D_2-dopamine receptors (224,225), but not by α_{2B}-adrenergic receptors (226). Activation of phospholipase A_2 is shared by SSTR4 (136), D_2-dopamine receptors (227), and α_{2B}-adrenergic receptors (228); however, inhibition of NHE1 is associated with both increases (229) and decreases (230) in phospholipase A_2 activity. One conserved function shared by all three receptor types is the

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inhibition of endocrine secretion and neurotransmitter release (97,231-233). Although a specific role for NHE1 activity in secretion has not been determined, remodeling of the actin-based cytoskeleton, which is impaired by inhibition of NHE1 (157,164,174,175), is a critical determinant in the secretory process (234,235).

In summary, we identified specific motifs in the IL2 and IL3 domains of GPCRs from distinct receptor families that may confer a common action of inhibiting NHE1 activity. The coordinate requirement of these motifs for inhibiting NHE1 suggests a cooperative interaction of IL2 and IL3 domains for effector coupling. Moreover, our findings identify a molecular basis for selective signaling by distinct receptor subtypes that are activated by common ligands.

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CHAPTER III

SOMATOSTATIN, ACTING AT RECEPTOR SUBTYPE SSTR1, INHIBITS Rho ACTIVITY AND THE ASSEMBLY OF ACTIN STRESS FIBERS AND FOCAL ADHESIONS

The low molecular weight GTPase Rho plays a central role in regulating organization of the actin-based cytoskeleton in mammalian cells. Activated, GTP-bound Rho promotes the formation of contractile actin filaments into stress fibers and the assembly of cell adhesion complexes (236,237). Rho is activated by transmembrane receptors, including the integrin family of adhesion receptors (238) and a subset of heptahelical G protein-coupled receptors (GPCRs) (239). While the signaling pathway linking integrin receptors to Rho has not been determined, GPCRs, including those for lysophosphatidic acid (LPA) (240-242) and thrombin (241,243,244), activate Rho through the heterotrimeric GTPases $G\alpha_{12}$ and $G\alpha_{13}$. GPCRs linked to the inhibition of Rho and downstream cytoskeletal reorganization, however, have not been identified.

We now report that somatostatin (SST), acting at the GPCR receptor subtype SSTR1, inhibits Rho activity and attenuates the assembly of actin stress fibers and focal adhesions. Five distinct SSTR subtypes have been identified that regulate a number of cellular functions, including endocrine and exocrine secretion, water and electrolyte transport, cell proliferation, and neuronal activity (97,100). Effector pathways regulated by SSTRs, including inhibition of adenylate cyclase and Ca^{2+} channel activity and stimulation of K^+ channel and

phosphatase activity are mediated by pertussis toxin (PTX)-sensitive mechanisms, most likely involving GTPases of the $G\alpha_i$ family (97,100).

The rationale for studying SST effects on Rho and the cytoskeleton was based on our previous studies with the Na-H exchanger, NHE1. NHE1 plays a critical role downstream of Rho in regulating cytoskeletal organization (175). NHE1 is phosphorylated directly by the Rho effector kinase p160ROCK (174), and activation of NHE1 by GPCRs, such as those for LPA and thrombin, and by integrin receptors is mediated by Rho and ROCK (164,174). Moreover, through its direct association with the ERM (ezrin, radixin and moesin) family of actin-binding proteins, NHE1 acts as a plasma membrane anchor for actin filaments to control the assembly of cortical stress fibers and focal adhesions (157). In contrast to LPA and thrombin, which stimulate Rho and NHE1, SST, acting at the SSTR1 but not the SSTR2 subtype, inhibits NHE1 activity (109). We reasoned that inhibition of NHE1 by SSTR1 might be associated with an inhibition of Rho.

Consistent with this rationale, findings from the current study indicate that SSTR1, but not SSTR2, stably expressed in fibroblasts inhibits Rho activation and cytoskeletal reorganization by thrombin and integrin activation. Moreover, in contrast to most effects of SSTRs which are abolished by PTX, SSTR1 inhibition of NHE1 (109) and, as we now report, Rho activity, is PTX-insensitive.

INVENTION

MATERIALS AND METHODS

Cell culture— CCL39 hamster lung fibroblasts were maintained in Dulbecco's modified Eagle's medium containing 5% fetal calf serum (FCS). The human *h*SSTR1 and *h*SSTR2 were stably expressed in CCL39 cells using the inducible mammalian expression vector pCMV (109). Cells were co-transfected with pRSV-neo using calcium phosphate precipitation and G418-resistant clones were selected and examined for their ability to bind [^{125}I-Tyr^{11}]SST-14 and mediate SST inhibition of cAMP accumulation, as previously described (109). To determine sensitivity to PTX, cells were pretreated with 100 ng/ml of the toxin (List Biologicals) for 18 h.

Affinity-detection of GTP-Rho — The abundance of activated, GTP-bound Rho was determined by a modification of previously described methods (238,242), using affinity adsorption with a GST fusion protein of the Rho-binding domain of Rho-kinase (amino acids 934-1015 of p160ROCK, kindly provided by S. Narumiya). The BL21 bacterial strain was transformed with this construct, and expression of the fusion protein was induced by 0.5 mM isopropyl-1-thio-β-D-galactopyranoside for 3 h at 37°C. Bacteria were lysed in buffer containing 50 mM Tris, pH 7.5, 150 mM NaCl, 5 mM $MgCl_2$, 1mM dithiothreitol, and 1% Triton X-100. Bacterial lysates were sonicated with four 15-s pulses, and then cleared by centrifugation at 21,000 x *g* for 30 min, after which the fusion protein was recovered by addition of glutathione beads to the supernatant.

Invalid

Fibroblasts, plated at a density of 1 X 10^6 in 100-mm plates, were maintained in medium containing 5% FCS for 24 h, washed, and then maintained for an additional 24 h in medium containing 0.5% FCS. Cells were treated with 30 nM thrombin (Enzyme Research Labs) in the absence or presence of 100 nM SST (Bachem) for the indicated times. To determine the response to integrin activation, quiescent cells were trypsinized, diluted with DMEM containing trypsin inhibitor (50 μg/ml; Sigma), collected by centrifugation, and resuspended in DMEM in the absence of FCS. Cells were then plated in the absence or presence of SST for 60 min in 100-mm plates coated with poly-L-lysine (10 μg/ml; Sigma) or fibronectin (20 μg/ml; Sigma). After washing three times in ice-cold phosphate buffered saline (PBS) containing 1 mM orthovanadate, cells from each 100-mm plate were lysed in 400 μl lysis buffer (50 mM Tris pH 7.2, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 500 mM NaCl, 10 mM $MgCl_2$, 10μg/ml leupeptin and aprotinin, and 1mM phenylmethylsulfonyl fluoride. The lysates from 2-3 plates were pooled, aspirated three times through a 26-gauge needle, and cleared by centrifugation at 13,000 x *g* 4°C for 3 min. A 40-μl aliquot of the supernatant was collected for determination of total cellular Rho. The remaining supernatant was added to 75μg GST-fusion protein-coated beads and incubated for 1 h at 4°C on a rotator. The beads were washed four times with wash buffer (Tris with 1% Triton X-100, 150 mM NaCl, 10 mM $MgCl_2$, 10μg/ml leupeptin, 10μg/ml aprotinin, and 1 mM phenylmethylsulfonyl fluoride), and proteins were separated by 12% SDS-PAGE. GTP-bound and total cellular Rho were detected by Western blotting using a monoclonal antibody to Rho-A

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(Transduction Laboratories) at a dilution of 1:250. Densitometry analysis was performed with NIH image, and the amount of GTP-bound Rho was expressed as a percentage of total cellular Rho.

Immunocytochemistry — Cells grown on 22-mm glass coverslips were maintained for 24h in 0.5% FBS and then treated with 30nM thrombin in the absence or presence of 100nM SST for 20 min. They were washed briefly with phosphate buffer and fixed in 2% paraformaldehyde at room temperature for 10 min. After thorough washing with PBS to remove excess fixative, the cells were permeabilized for 10 min in 0.1% Triton X-100; immersed in 5% normal calf serum; and incubated with primary antibodies, including those against paxillin (1:200 for 1 h at 23°C; Zymed Laboratories Inc.), and RhoA (1:250 for 48 h at 4°C; Transduction Laboratories). The bound antibodies were detected using the relevant fluorescein isothiocyanate-conjugated secondary antibody (either goat anti-rabbit IgG or donkey anti-mouse IgG, Jackson ImmunoResearch Laboratories, Inc.) at a dilution of 1:500 for 1h at room temperature. The coverslips were mounted on glass slides with Vectashield anti-fade mounting medium (Vector Labs, Inc.) and screened using a Zeiss Axiophot microscope (magnification X1000). Representative images were collected using a Spot2 camera (Diagnostic Instruments, Inc.) and imported into Adobe Photoshop.

Rho translocation — The subcellular localization of Rho was determined by immunostaining and immunoblotting. For immunoblotting, cells plated on 100-

mm plates were maintained for 24 h in 0.5 % FCS and then treated with thrombin (30 nM) in the absence or presence of SST (100 nM) for 20 min. Cells were washed twice with PBS, collected by scraping in buffer containing 50 mM Hepes, 135 mM NaCl, 3 mM KCl, and 3 mM EDTA, pH 7.5, and lysed by sonication. Post-nuclear supernatants were obtained by centrifugation and separated into S100 and P100 fractions by centrifugation at 100,000 x *g* for 15 min. Proteins were separated by SDS-PAGE, transferred to nitrocellulose membranes, and probed with antibodies to Rho (Santa Cruz Biotechnology).

RESULTS

SSTR1, but not SSTR2, inhibits actin stress fiber assembly induced by thrombin — Our initial studies investigated whether SST regulates stress fiber assembly in CCL39 fibroblasts stably expressing *h*SSTR1 or *h*SSTR2. Receptor expression was determined by membrane binding to [^{125}I-Tyr^{11}]SST-14 (109), and receptor function was confirmed by the ability of both receptor subtypes to mediate a PTX-sensitive inhibition of cAMP accumulation by SST (Fig. 11A).

Treating quiescent CCL39 cells with thrombin (30 nM; 20 min) resulted in the assembly of long parallel arrays of actin stress fibers that extended throughout the cell (Fig. 11B), although the morphology of the cells was not markedly altered. In the presence of SST (100 nM), thrombin-induced stress fiber formation was unchanged in wild-type CCL39 cells and in CCL39 cells expressing SSTR2 (CCL39-R2), but was strikingly inhibited in CCL39 cells expressing SSTR1 (CCL39-R1) (Fig. 11B). Actin stress fibers were absent in the cell body, and their abundance and size were markedly decreased in the cortex.

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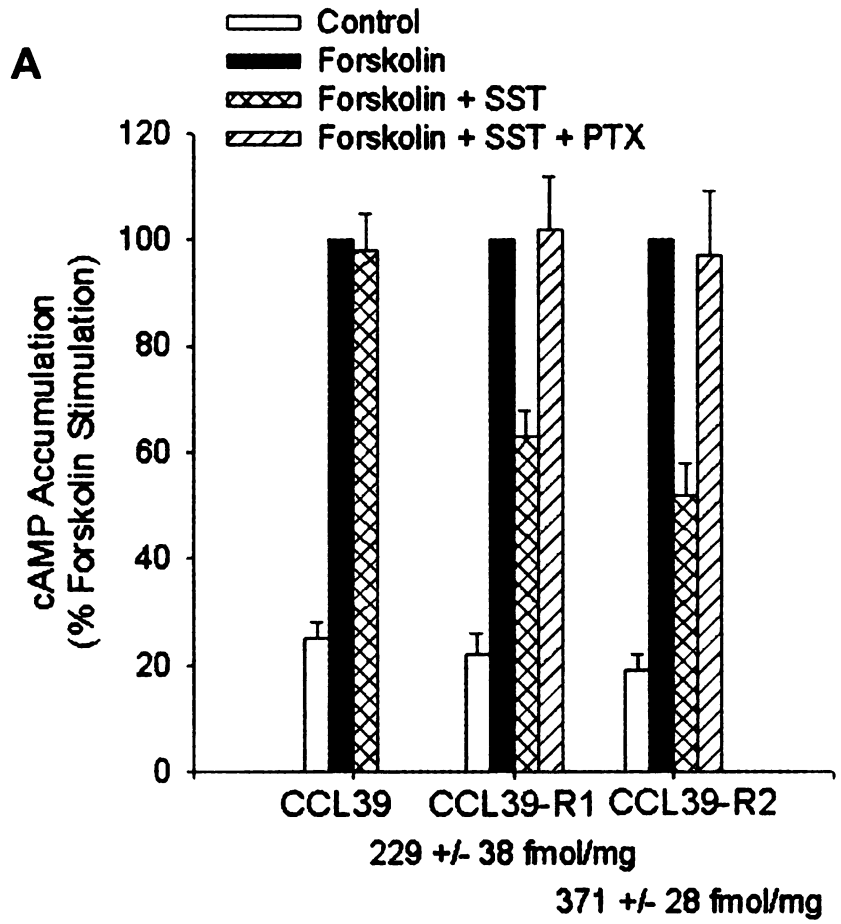

FIG 11. **Somatostatin inhibits cAMP accumulation and actin stress fiber assembly**. (A) cAMP accumulation was determined in wild-type CCL39 cells and in cells stably expressing *h*SSTR1 (CCL39-R1) and *h*SSTR2 (CCL39-R2). Data are expressed as percentage of forskolin (10 μM) stimulation. Also included are data of cAMP accumulation by forskolin (10 μM) plus SST (100 nM) with and without pretreament with PTX (100 ng/ml for 18 h). Forskolin-induced increases in cAMP accumulation were not significantly different in the absence and presence of PTX (data not shown). Expression of SSTR1 and SSTR2 was determined by radioligand binding of cell membranes with [^{125}I-Tyr^{11}]SST-14 and expressed as fmol/mg protein. Data represent the means ± S.D. of three separate cell preparations for cAMP accumulation, and the means of four separate membrane preparations for radioligand binding. (B) Phalloidin labeling of actin filaments in quiescent cells (control) and cells treated with thrombin in the absence and presence of SST (shown in next page). Images are representative of more than 80% of the total population of cells in four separate preparations.

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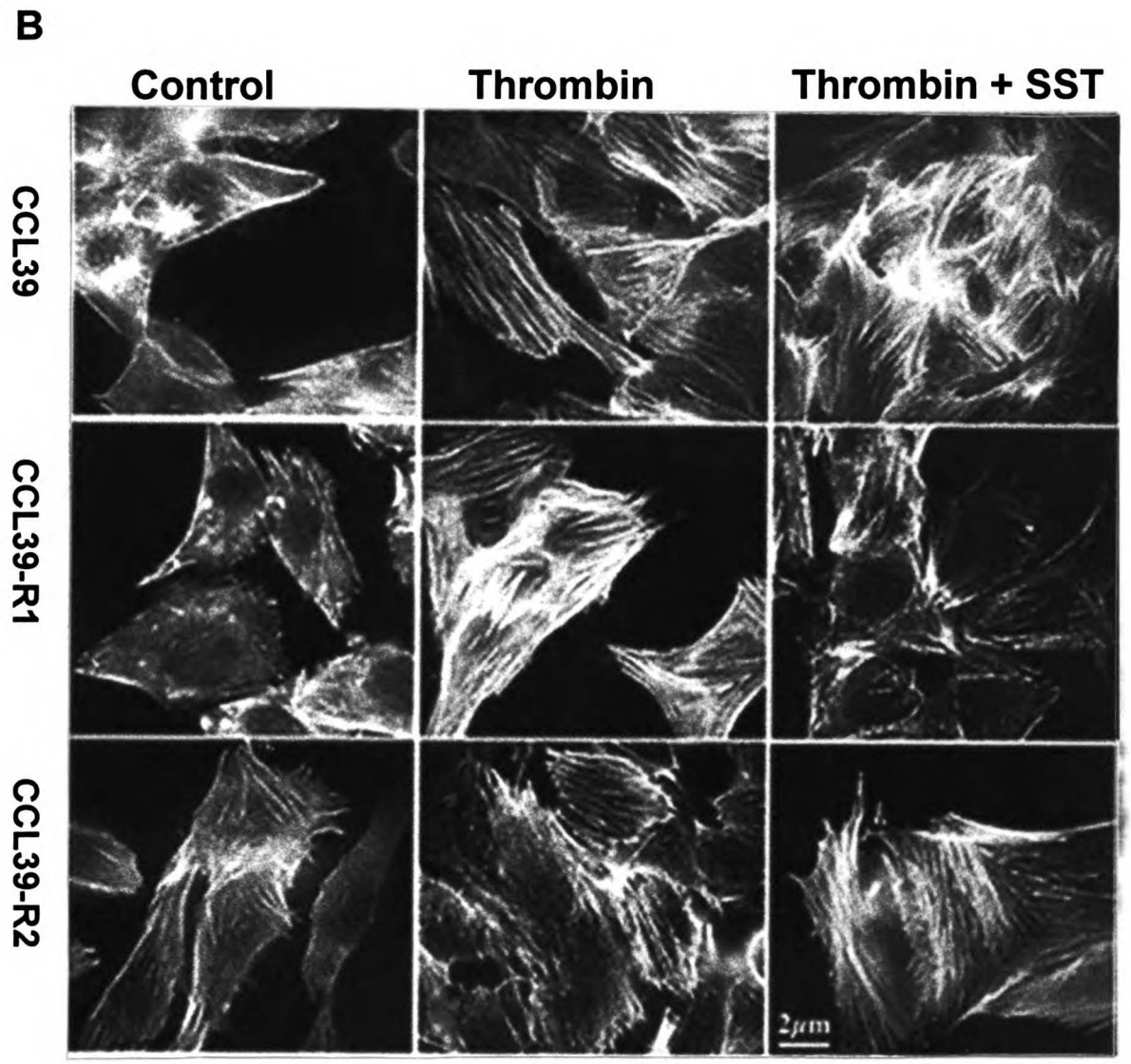
B
Control
Thrombin
Thrombin + SST
CCL39
CCL39-R1
CCL39-R2
2μm

SSTR1, but not SSTR2, inhibits Rho activation induced by thrombin — To determine whether SSTR1 attenuation of stress fiber assembly was associated with an inhibition of Rho activity, we measured the abundance of GTP-bound Rho complexed to a GST-fusion protein of the Rho-binding domain of its effector, ROCK. Thrombin (30 nM) increased the abundance of Rho-GTP, with maximal stimulation occurring at 10-20 min. Activation decreased between 30-60 min, but higher than in control cells (data not shown). In the presence of SST (100 nM), maximal Rho activation by thrombin at 20 min was inhibited by 75% in CCL39-R1 cells, but was unchanged in wild-type CCL39 cells and in CCL39-R2 cells (Fig. 12A). In the absence of thrombin, SST had no effect on the abundance of Rho-GTP in all three cell types (data not shown), indicating that SSTR1 inhibits stimulated, but not basal, Rho activity.

Although most effector actions of SST are abolished by PTX, SST inhibition of NHE1 is PTX-insensitive (109,126). Consistent with this finding, the inhibition of Rho activity and stress fiber assembly by SST was not blocked by pre-incubation with PTX (Fig. 12B), although the inhibition of cAMP accumulation was completely reversed (Fig. 11A). In PTX-treated cells, however, basal and thrombin-stimulated Rho-GTP levels were consistently less than in untreated cells, suggesting that PTX attenuated or blunted the activation of Rho.

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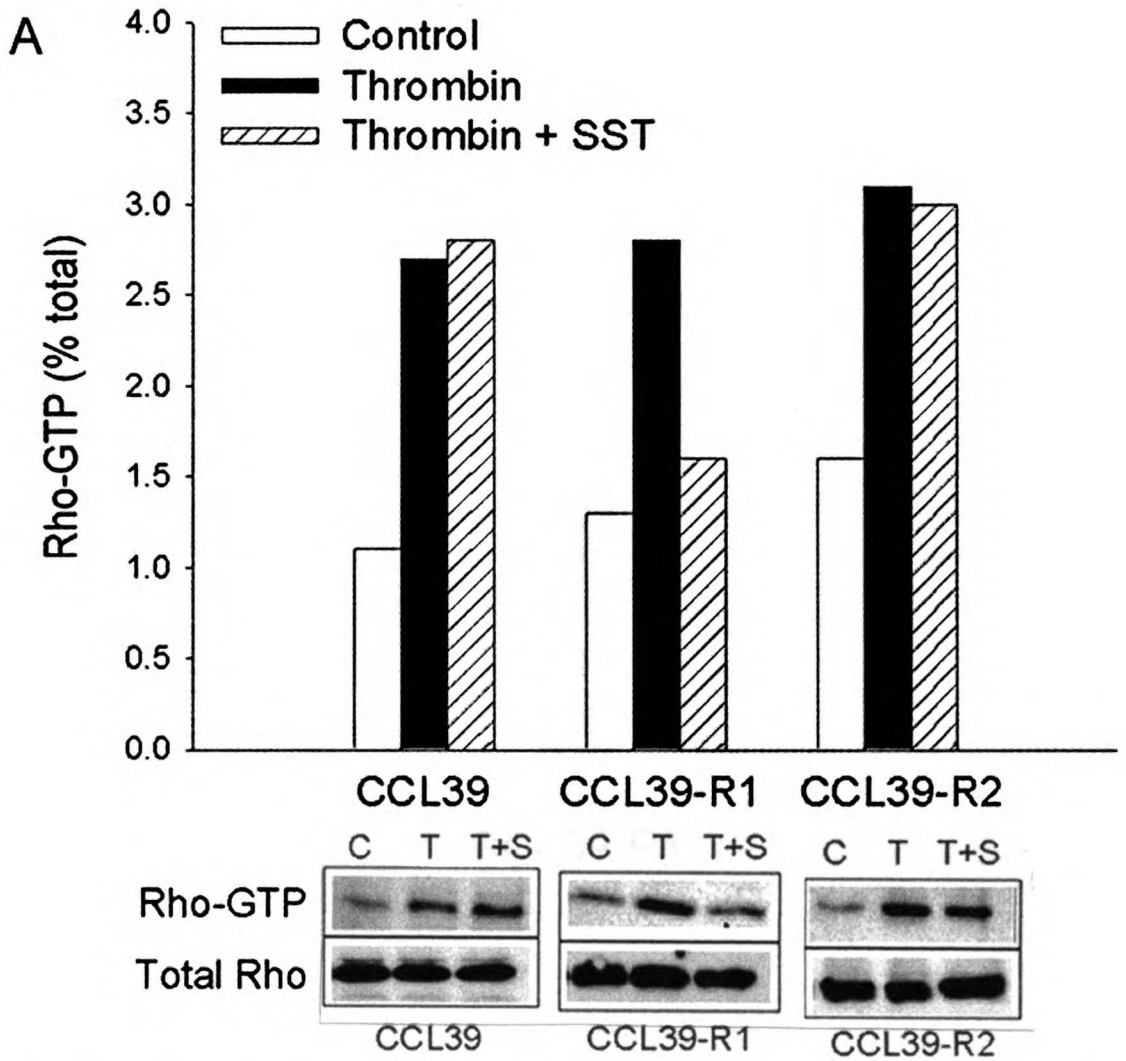

FIG 12. **Somatostatin inhibits activation of Rho by thrombin**. The abundance of GTP-bound Rho complexed with the Rho-binding domain of ROCK and total Rho in post-nuclear supernatants was determined by immunoblotting, and the abundance of Rho-GTP was expressed as a percentage of total Rho immunoreactivity. (A) The abundance of Rho-GTP and immunoblots of Rho-GTP and total Rho acquired from wild-type CCL39 cells and CCL39-R1 and CCL39-R2 cells. Data were obtained from quiescent cells (control) and cells treated with thrombin (30 nM; 20 min) in the absence and presence of SST (100 nM), and are representative of five separate cell preparations. (B) The abundance of Rho-GTP and immunoblots of Rho-GTP and total Rho obtained from control and ligand-treated CCL39-R1 cells pretreated with PTX (100 ng/ml) for 18 h (shown in next page). Data are representative of three separate cell preparations.

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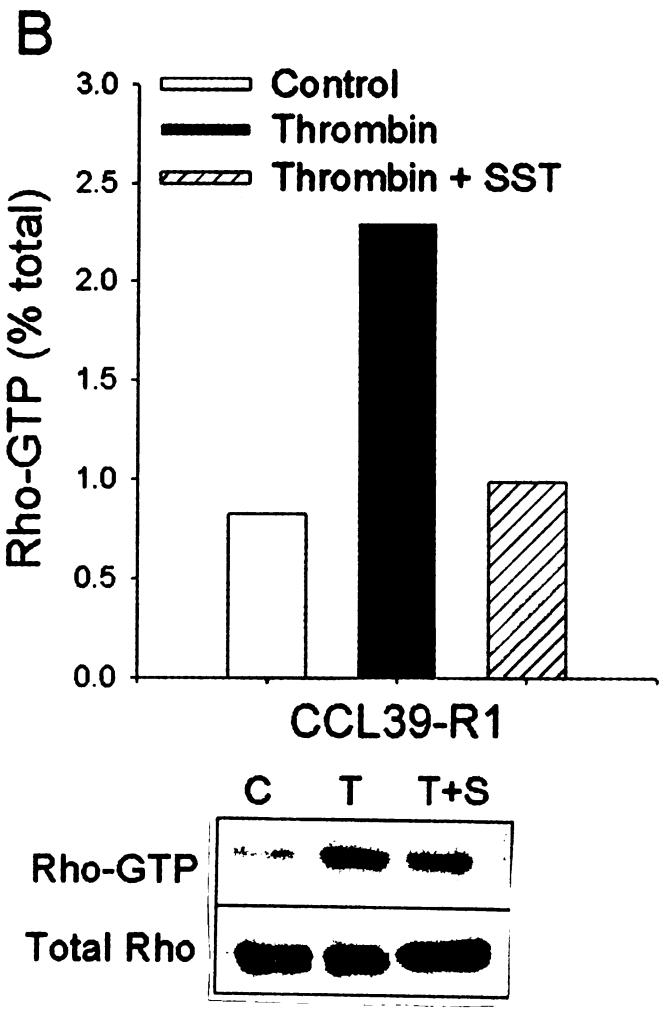
B
Control
Thrombin
Thrombin + SST
Rho-GTP (% total)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
CCL39-R1
C
T
T+S
Rho-GTP
Total Rho

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Because activation of Rho is associated with its translocation from the cytoplasm to the plasma membrane, we determined whether changes in Rho translocation were associated with the SSTR1-mediated decrease in Rho-GTP. Immunoblotting for Rho indicated that thrombin increased the abundance of Rho in the P100 fraction of CCL39-R1 cells, compared to quiescent (control) cells (Fig. 13A). In the presence of SST, the thrombin-induced abundance of membrane-associated Rho was reduced by 50%. The caveat of using subcellular fractionation to determine protein localization is that the P100 fraction does not reveal which membranes are involved. We therefore used immunostaining to determine whether changes in the abundance of Rho in the P100 fraction reflected changes in its localization at the plasma membrane. In thrombin-treated CCL39 and CCL39-R1 cells, the distribution of Rho immunoreactivity shifted from a predominant intracellular localization seen in quiescent cells to more marked staining at the cell membrane (Fig. 13B). Pretreatment with SST blocked this redistribution of Rho immunoreactivity in CCL39-R1 cells, but not in wild-type CCL39 cells, indicating that SSTR1 inhibition of Rho-GTP is also associated with an inhibition in its translocation to the plasma membrane.

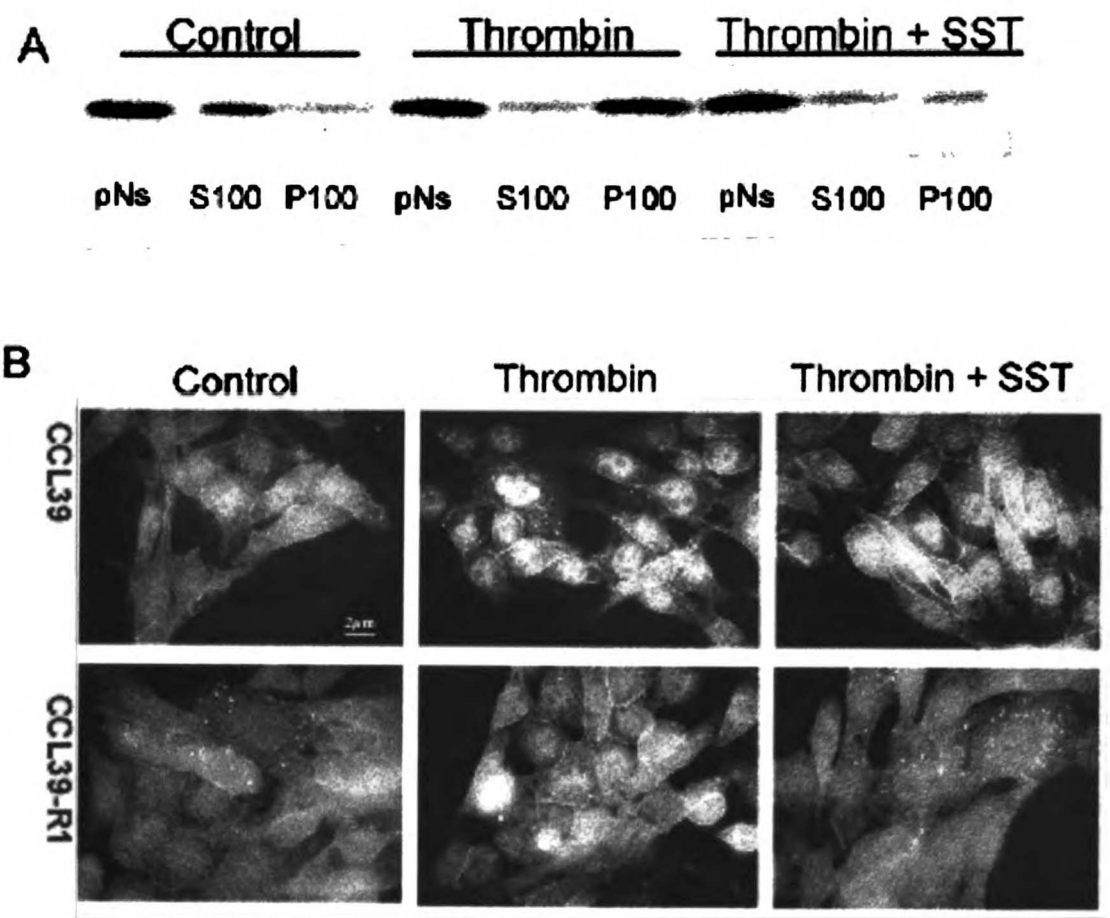

FIG 13. **Somatostatin inhibits the membrane-associated abundance of Rho immunoreactivity in response to thrombin.** (A) Immunoblots of Rho abundance, respectively, in post-nuclear supernatants (pNs) and in S100 and P100 fractions obtained from control CCL39-R1 cells and CCL39-R1 cells treated with thrombin (30 nM) in the absence and presence of SST (100 nM). Data represent one-tenth of the total post-nuclear supernatants, one-fourth of the S100 fraction, and one-half of the P100 fraction and were similar in three separate cell preparations. (B) Immunostaining for Rho in control CCL39-R1 cells and CCL39-R1 cells treated with thrombin (30 nM) in the absence and presence of SST (100 nM). Images are representative of >80% of the cells in three separate cell preparations.

SSTR1 inhibits Rho activity and focal adhesion assembly by integrins — In addition to its central role in mediating GPCR regulation of cytoskeletal reorganization, Rho is also activated by integrin receptors, and it regulates integrin-induced assembly of focal adhesions. CCL39 cells express $\alpha_5\beta_1$ integrins, which can be activated by plating the cells on fibronectin to promote the assembly of actin stress fibers and paxillin-rich focal adhesions (164). To determine whether SSTR1 also inhibits activation of Rho by integrins, we determined the effects of SST in CCL39-R1 and CCL39-R2 cells plated on fibronectin.

Compared to control cells plated on poly-L-lysine, plating on fibronectin for 60 min induced an increase in Rho activity in both cell types. Although preincubating cells with SST for 5 min prior to plating had no effect on cell attachment (data not shown), it inhibited Rho activation by fibronectin in CCL39-R1 cells, but not in CCL39-R2 cells (Fig. 14A). Moreover, SST treatment had a dramatic effect on the assembly of focal adhesions and stress fibers by fibronectin in CCL39-R1 cells. In the absence of SST, immunostaining showed that the focal adhesion-associated protein paxillin was localized in densely packed bundles within peripheral focal adhesions, and phalloidin staining revealed densely packed actin filaments predominantly at the cortex (Fig. 14B). With SST treatment, however, paxillin immunostaining revealed smaller, punctate focal complexes, indicating impaired assembly of focal adhesions (Fig. 14B). Additionally, in the presence of SST, fibronectin-induced stress fiber formation was dramatically inhibited in both the cell body and cortex (Fig. 14B). Hence,

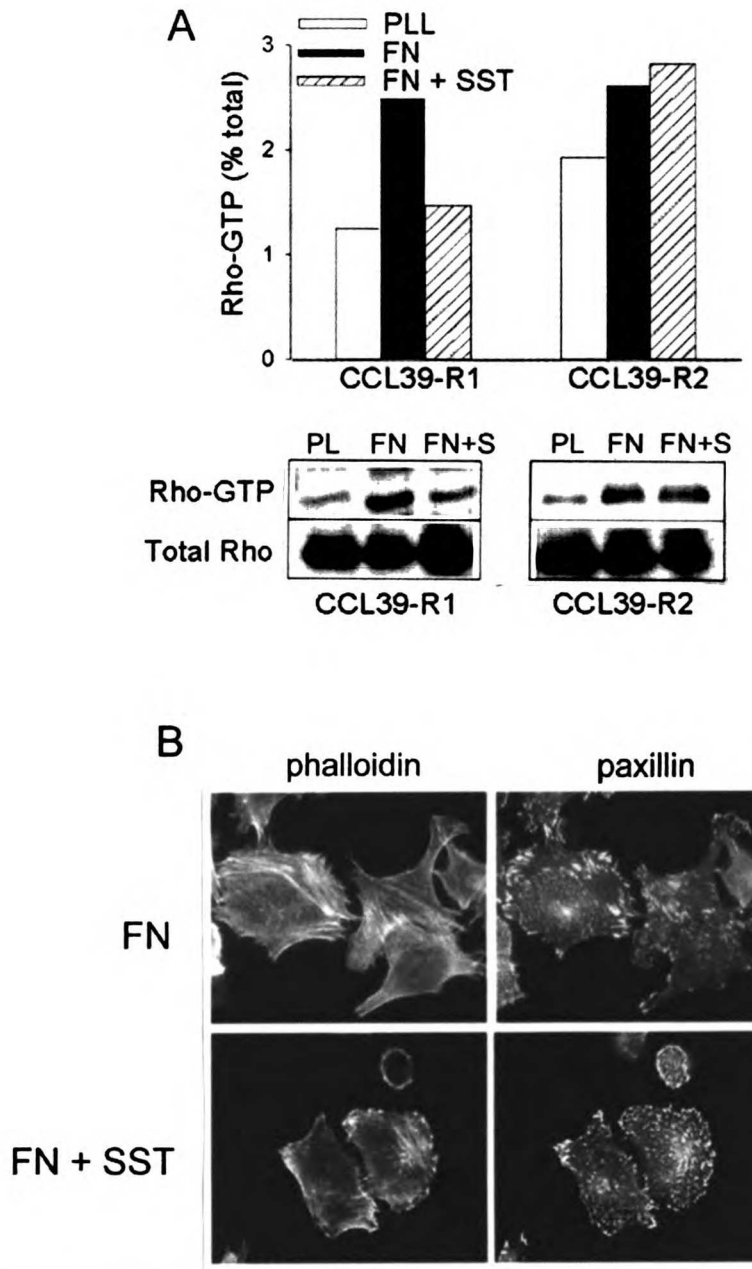

FIG 14. **Somatostatin inhibits activation of Rho and the assembly of focal adhesion in response to integrin activation.** (A) The abundance of GTP-bound Rho complexed with the Rho-binding domain of ROCK and total Rho in CCL39-R1 and CCL39-R2 cells was determined by immunoblotting, and the abundance of Rho-GTP is expressed as a percentage of total Rho immunoreactivity. Data were obtained from cells plated for 60 min on poly-L-lysine (PLL) (control) or fibronectin (FN) in the absence or presence of SST and are representative of three separate cell preparations. (B) The abundance of actin filaments, determined by phalloidin labeling, and the abundance of focal adhesions, determined by paxillin staining, are shown for CCL39-R1 cells plated for 60 min on fibronectin in the absence and presence of SST. Images are representative of >85% of the cells observed in four separate preparations.

DISCUSSION

The current study demonstrates for the first time that a GPCR, namely SSTR1, inhibits activation of Rho and that this effect is correlated with a decrease in actin stress fiber assembly. This effect was not shared by SSTR2. Somatostatin receptors have been subdivided based on sequence homology into two subgroups: SSTR1 and 4, and SSTR2, 3 and 5. The major functions of the latter group are well known because most commonly available somatostatin analogs show a higher affinity for members of the second group, including octreotide, which is widely used in a number of clinical settings (81,82). The functions of SSTR1 and 4 remain unclear because until recently (245-247) there were no receptor-specific analogs for members of this subgroup.

The SSTR family shows a widespread and overlapping cellular expression pattern, with many cell lineages expressing two or more subtypes. Most cells express SSTR2 and/or SSTR5 as well as SSTR1 and/or SSTR4, making it difficult to discriminate precisely which receptor is responsible for a given biological function without the aid of receptor-specific analogs. The consequence of multiple receptor subtype expression becomes particularly problematical when the formation of heterodimers between different SSTRs is taken into consideration (94,248). To circumvent this problem we investigated receptor-specific signaling by using hamster lung CCL39 fibroblasts stably expressing either *h*SSTR1 or *h*SSTR2.

In CCL39 fibroblasts stimulated by thrombin, parallel arrays of actin stress fibers were seen throughout the cytoplasm, as noted previously in fibroblasts

(241,249) and endothelial cells (250,251) and consistent with the activation of Rho (236). Although thrombin-stimulated endothelial cells undergo rounding (251), no change in the overall shape of the CCL39 cells was noted in the present experiments. Treatment of thrombin-stimulated cells with SST attenuated stress fiber formation in CCL39-R1, but not in wild-type CCL39 or in CCL39-R2, fibroblasts. This is consistent with a previous finding that SST decreases the abundance and size of actin stress fibers in HUVEC expressing endogenous SSTR1 (252).

Our data indicate that the inhibitory effect of SSTR1 on stress fiber assembly and Rho activation was not mediated by $G\alpha_{i/o}$ because PTX failed to prevent this effect. However, PTX treatment attenuated cAMP accumulation by SST and, as we previously determined, completely ADP-ribosylates PTX substrates (109). Although a number of GPCRs are known to activate Rho via $G\alpha_{12}$ and $G\alpha_{13}$ (241,243,244), our data are the first to demonstrate a GPCR able to inhibit Rho activation.

Thrombin stimulates Rho-GTP formation via $G\alpha_{12}$/ $G\alpha_{13}$, which activates a Rho-specific guanine nucleotide exchange factor promoting exchange of GTP for GDP (239). It is possible that SSTR1 activation promotes the association of Rho with a Rho GTPase-activating protein (RhoGAP) such as p190RhoGAP (253), thus reversing the activation caused by thrombin. Activation of SSTR1 might also stabilize the association of Rho with a Rho guanine dissociation inhibitor (GDI) (254) that prevents translocation to the plasma membrane and GTP binding. An interesting alternative possibility is that SSTR1 inhibits the activation

of $G\alpha_{12}$ or $G\alpha_{13}$ by thrombin, particularly because SST inhibited thrombin-stimulated, but not quiescent, Rho activity.

Inhibition of thrombin-activated Rho by SST was coupled with a decrease in the amount of Rho translocated to the plasma membrane, which suggests that RhoGDI might be involved in this response. Two CAAX domains in Rho are thought to mediate lipid anchoring, and in the GDP-bound conformation these domains may be masked, preventing the association of Rho with the plasma membrane (255).

Immunocytochemical studies in resting and SST-treated CCL39-R1 cells indicated that while some Rho immunoreactivity was present as a diffuse stain in the cytoplasm, a proportion was associated with small vesicular structures scattered throughout the cell with little evidence of plasma membrane, Golgi or nuclear staining. Overexpression of Green Fluorescent Protein-tagged RhoA and B in COS-1 and MDCK cells demonstrated that Rho-A in quiescent cells was predominantly present as a diffuse cytoplasmic fluorescence with no discernable plasma membrane staining, whereas RhoB was localized to the plasma membrane and Golgi stack (255). The staining obtained with CCL39-R1 cells is consistent with the localization of RhoA in the MDCK and COS-1 cells.

In addition to its central role in mediating GPCR regulation of cytoskeletal reorganization, Rho is stimulated by plating cells on a fibronectin substrate and activation of integrin receptors (238). In the present study the ability of SSTR1 to inhibit Rho activation was not limited to GPCR-activated pathways because integrin-stimulated Rho was attenuated by SST treatment. Again, this was

specific to CCL39-R1 cells; no effect of SST treatment was observed in CCL39-R2 cells. We previously reported that CCL39 cells express $\alpha_5\beta_1$ integrins, which can be activated by plating the cells on fibronectin to promote the assembly of actin stress fibers and paxillin-rich focal adhesions (164,174). In CCL39-R1 cells plated on fibronectin in the presence of SST, the number and size of the stress fibers and focal adhesions was reduced, consistent with the inhibition of Rho.

In summary, our data show for the first time that activation of a GPCR, SSTR-1, inhibits the GTP binding of Rho. The decrease in activated Rho was correlated with a decreased assembly of actin stress fibers.

Summary

The neurohumoral peptide somatostatin binds to five receptor subtypes (SSTR1-5) to inhibit a number of processes, including hormone secretion, cell proliferation, and tumor metastasis. All SSTR are heptahelical G protein-coupled receptors linked to the inhibition of adenylyl cyclase; however, distinct effects of different SSTR subtypes are suggested to be mediated by adenylyl cyclase-independent mechanisms. One adenylyl cyclase-independent action of somatostatin is to inhibit the plasma membrane Na-H exchanger, NHE1, and lower intracellular pH (pH_i), which could mediate inhibitory actions of SSTR. In this study, we determined which of the SSTR subtypes couple to NHE1 and identified receptor motifs essential for this coupling. To determine which SSTR subtypes inhibit NHE1, we stably expressed SSTR1-5 in clonal CCL39 fibroblasts. Receptor expression was verified by radioligand binding and function was confirmed by the ability of all SSTR to inhibit adenylyl cyclase. We found that SSTR1, 3, and 4, but not SSTR2 and 5, inhibit NHE1 and lower pH_i. Using chimeric SSTR2/SSTR1 receptors, we also determined that substitution of intracellular loops IL2 and IL3 of SSTR1 into SSTR2 conferred the ability to inhibit NHE1. Sequence alignment of IL2 and IL3 for all cloned SSTR revealed consensus motifs of T(S,P)V in IL2 and (W)QQ(R) in IL3 of SSTR1, 3, and 4 that are not present in SSTR2 and 5. Using site-directed mutagenesis, we replaced cognate amino acids in IL2 and IL3 of SSTR2 with TV and WQQ, respectively, and found that substitutions conferred the ability to inhibit NHE1 and acidify pH_i. Interestingly, identical motifs are found in IL2 and IL3 of other receptors coupling

to the inhibition of NHE1, such as the D2-dopamine receptor and α2-adrenergic receptor type B, but not in receptors positively regulating NHE1, such as the thrombin receptor, α1-adrenergic receptor, and β2-adrenergic receptor. Our findings demonstrated subtype-selective coupling of SSTR to NHE1 and, moreover, identified receptor consensus motifs essential for this coupling. Because the GTPase $G\alpha_i$ that couples SSTR to adenylyl cyclase does not signal to NHE1, it is likely that the consensus motifs we identified confer signaling to a distinctly different GTPase.

Somatostatin regulates multiple biological functions acting through a family of five G-protein coupled receptors, SSTR1-5. While all five receptors inhibit adenylate cyclase activity and decrease intracellular cAMP levels, individual receptors have been shown to couple to additional signaling pathways. In CCL39 fibroblast cell lines expressing either hSSTR1 or hSSTR2 we demonstrate for the first time that activation of SSTR1, but not SSTR2, attenuated both thrombin-stimulated Rho-GTP complex formation. Activation of Rho results in the formation of intracellular actin stress fibers. In the CCL39 cells expressing SSTR1, somatostatin treatment prevented actin stress fiber formation. These data show for the first time that a G-protein coupled receptor, SSTR1, inhibits the activation of Rho thus preventing stress fiber formation.

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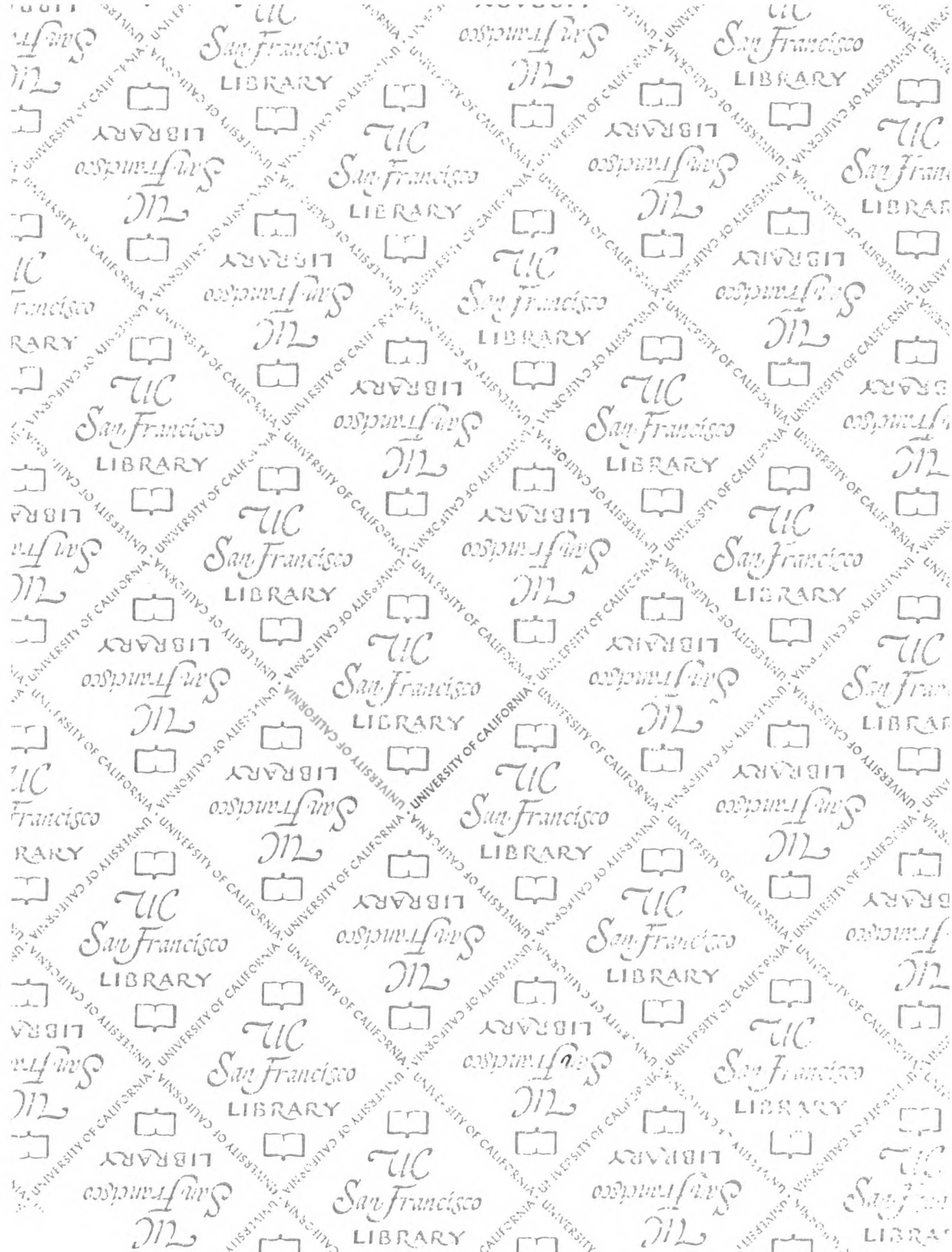
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