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Somatostatin receptor subtype SSTR2 mediates the inhibition of high-voltage-activated calcium channels by somatostatin and its analogue SMS 201-995

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Abstract Somatostatin and its anologue SMS 201-995 inhibit high voltage-activated (HVA) Ca^{2+} currents in the rat insulinoma cell line RINm5F which stably express cloned human somatostatin receptor subtype 2 (hSSTR2). In contrast, neither somatostatin nor SMS 201-995 suppresses the HVA Ca^{2+} currents in RINm5F which stably express cloned hSSTR1. These results suggest that somatostatin-induced inhibition of HVA Ca^{2+} currents is mediated by a specific receptor subtype and that inhibition of calcium influx through HVA Ca^{2+} channels is one of the mechanisms of SMS 201-995 action on inhibitory processes of hormone secretion and cell proliferation.

Key words: Somatostatin analogue; Somatostatin receptor subtype; Voltage-dependent calcium channel; G-protein; Intracellular calcium

1. Introduction

Somatostatin, a tetradecapeptide originally discovered as a hypothalamic inhibitor of growth hormone secretion [1], exerts diverse biological actions in many other tissues, including inhibition of secretory and proliferative processes [2,3]. The biological functions of somatostatin are mediated by its specific receptors which are coupled to GTP-binding (G) proteins [4]. Various somatostatin analogues have been developed for clinical applications to inhibit hormone secretion and cell proliferation [5]. One of the somatostatin analogues, SMS 201-995, has been approved for the treatment of endocrine tumors [6]. However, the mechanism of its action is still unknown. Intracellular calcium ($[Ca^{2+}]_i$) is the principal signal for many cellular functions, including hormone secretion and cell proliferation [7,8]. Although somatostatin has been shown to inhibit calcium influx through voltage-dependent calcium channels (VDCC) [9–12], the effect of SMS 201-995 on VDCC is not yet evaluated. Recently, we have identified five subtypes of human somatostatin receptor (hSSTR1–5), the mRNAs of which are expressed in a tissue-specific manner [13–15]. Of these, hSSTR2 has been shown to have the highest binding affinity for SMS 201-995 [16]. In addition, we have found that the efficacy of SMS 201-995 in the treatment of endocrine tumors may depend, at least in part, on the expression of hSSTR2 in the tumors [16]. In this report, we show that both somatostatin and SMS 201-995 inhibit high-voltage-activated (HVA) Ca^{2+} currents in insulin-secreting RINm5F cells stably expressing hSSTR2, while somatostatin does not inhibit HVA Ca^{2+} currents in RINm5F cells expressing hSSTR1, suggesting that inhibition of calcium influx through VDCC, which is mediated by the specific receptor subtype hSSTR2, could contribute in part to the suppressive effects of SMS 201-995 on hormone secretion and cell proliferation in the treatment of endocrine tumors.

2. Materials and methods

2.1. Cell culture

RINm5F cells were grown in monolayers in Dulbecco's modified Eagle medium (DMEM) containing 10% fetal bovine serum (Gibco/BRL) in an atmosphere of 5% CO_2 and 95% air at 37°C.

2.2. Establishment of RINm5F cells stably expressing human somatostatin receptors

A *PstI/XmnI* fragment of the hSSTR1 gene [13], and a *BamHI/XbaI* fragment of the hSSTR2 gene [13] were inserted into mammalian expression vector, pCMV6b. RINm5F cells stably expressing hSSTR1 or hSSTR2 were established as previously described [13]. Briefly, the expression plasmids were cotransfected with pSV2neo into RINm5F cells using Lipofectin reagent (Gibco/BRL). Stable transformants were selected in the culture medium containing G418 at 400 μg/ml. For control cells, RINm5F cells transfected with plasmid vector alone were used.

2.3. RNA blot analysis

Total cellular RNA was isolated from transfected and untransfected RINm5F cells by guanidinium isothiocyanate/CsCl procedure. For RNA transfer blots, 20 μg of total cellular RNA were denatured with formaldehyde, electrophoresed on 1% agarose gel, and transferred to a nylon membrane. Membranes were hybridized with a ^{32}P-labeled *PstI/XmnI* (nucleotide −98 ~ +1400, relative to the translation start site) hSSTR1 or *BamHI/XbaI* (nucleotide −81 ~ +1215) hSSTR2 gene probe using high-stringency conditions (42°C; 50% formamide, 0.75 M NaCl/75 mM sodium citrate, 2 × Denhardt's solution (Denhardt's solution = 0.02% bovine serum albumin/0.02% Ficoll/0.02% polyvinylpyrrolidone), 20 mM sodium phosphate buffer (pH 6.5), 0.1% NaDodSO$_4$, 100 μg of sonicated, denatured salmon testis DNA per ml, 10% dextran sulfate, and 3~4 × 10^6 cpm of probe per ml) [13].

2.4. Binding experiments

The incubation medium used for the binding experiments was a mixture of 1 mg/ml bacitracin, 0.1 mM *p*-amidinophenyl methanesulfonyl fluoride hydrochloride, 20 pM [^{125}I-Tyr^{11}]somatostatin-14 in the absence or presence of somatostatin and SMS 201-995 at various

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concentrations as indicated in Fig. 1B. After incubation for 1 h at 20°C, the cells were washed twice with the ice-cold phosphate-buffered saline and solubilized in 1 ml of 1 N NaOH, and the radioactivity was measured in a γ-counter.

2.5. Electrophysiological recordings

The whole-cell voltage clamp technique was carried out to record VDCC currents in RINm5F cells [17]. Ba^{2+} was used as a charge carrier for measurement of VDCC currents. The extracellular recording solution was a mixture of 20 mM $Ba(OH)_2$, 110 mM *N*-methyl-D-glucamine, 10 mM tetraethylammonium chloride, 140 mM methanesulfonate, 20 mM 4-aminopyridine, and 10 mM MOPS (pH 7.4). The pipette solution was a mixture of 140 mM cesium aspartate, 10 mM EGTA, 5 mM Mg-ATP, and 10 mM MOPS (pH 7.2). GTPγS was added to the pipette solution at a final concentration of 200 μM. Pertussis toxin (PTX) was added to the culture medium at a final concentration of 200 ng/ml for 12 h before recordings. After achieving the whole-cell configuration of the patch-clamp recording, the cells were maintained at a holding potential of −80 mV. VDCC currents were recorded by depolarizing the membrane to test pulse potentials from −40 mV to +80 mV in +10 mV steps at every 10 s. The effects of somatostatin and SMS 201-995 on peak VDCC currents were evaluated at a test potential of +20 mV. Test agents were dissolved in the extracellular solution and applied to a recording chamber (0.3 ml in volume) at a flow rate of 6 ml/min. The recordings were performed at room temperature (20–22°C). Values are expressed as mean ± S.E.M. Statistical analyses were conducted using Student's *t*-test.

2.6. cAMP measurement

The transfected RINm5F cells were split into 24-well plates and grown to confluency in the culture medium described above. The cells were washed with DMEM containing 1 mg/ml bovine serum albumin and incubated for 45 min at 37°C in 2 ml of the same buffer or the same buffer containing 1-methyl-3-isobutylxanthine (0.2 mM) with forskolin (1 mM) or a combination of forskolin and somatostatin (100 nM). cAMP levels were quantified by enzyme immunoassay system (Amersham) as described [18].

3. Results

3.1. Establishment of RINm5F cells stably expressing hSSTR1 and hSSTR2

RINm5F subclonal cells which most abundantly express the hSSTR1 (designated R1–7) or hSSTR2 (designated R2–14) were selected by RNA blot and binding analyses. As shown in Fig. 1A, RNA blot analysis revealed a 2.0 kb or a 1.8 kb transcript in R1–7 or R2–14 cells, respectively, whereas neither hSSTR1 nor hSSTR2 mRNA was detected in RINm5F cells transfected with plasmid vector alone.

3.2. Competitive binding experiments

To ascertain that hSSTR1 and hSSTR2 proteins are expressed in these cells, we performed competitive binding experiments. While no specific binding was detected in RINm5F cells transfected with vector alone, both R1–7 and R2–14 cells showed specific binding of [^{125}I-Tyr^{11}]somatostatin (see Fig. 1, legend). In competition binding experiments, somatostatin-14 potently inhibited [^{125}I-Tyr^{11}]somatostatin binding in R1–7 and R2–14 cells with a half-maximal inhibitory concentration (IC_{50}) value of 1.0 nM in both cells. SMS 201–995 inhibited [^{125}I-Tyr^{11}]somatostatin binding in R2–14 and R1–7 cells with an IC_{50} value of 13 nM and >1 μM, respectively (Fig. 1B).

3.3. Effects of somatostatin and SMS 201-995 on VDCC currents in hSSTR1-expressing and hSSTR2-expressing RINm5F cells

Since the VDCC currents detected in RINm5F cells used in the present study were reduced by the addition of the L-type Ca^{2+} channel blocker nifedipine (80.4 ± 3.0% of control currents at 5 μM, at a holding potential of −80 mV, n = 7) and were not reduced by the addition of the N-type Ca^{2+} channel blocker ω-conotoxin GVIA (96.5 ± 6.1% of control currents at 10 μM, n = 5), the VDCC currents found in RINm5F cells are HVA Ca^{2+} currents including L-type but not N-type. As shown in Figs. 2A,B and 3A, both somatostatin and SMS 201-995 significantly inhibited the HVA Ca^{2+} currents in R2–14 cells. The inhibitory effects of somatostatin and SMS 201-995 were voltage-dependent, as shown in Fig. 2E. The inhibition of the Ca^{2+} currents by these agents was most evident at test-pulse potentials between +10 and +40 mV. In addition to the reduction in the peak current amplitudes, the activation process of the currents was slowed by somatostatin and SMS 201-995 (Fig. 2A and B). The concentrations of somatostatin and SMS 201-995 for inhibition of the HVA Ca^{2+} currents were in reasonable agreement with potencies of these agents in inhibition of [^{125}I-Tyr^{11}]somatostatin binding in these cells. In contrast, neither somatostatin nor SMS 201-995 inhibited the HVA Ca^{2+} currents in R1–7 cells (Figs. 2C and 3A) or RINm5F cells transfected with plasmid vector alone (control cells) (Figs. 2D and 3A).

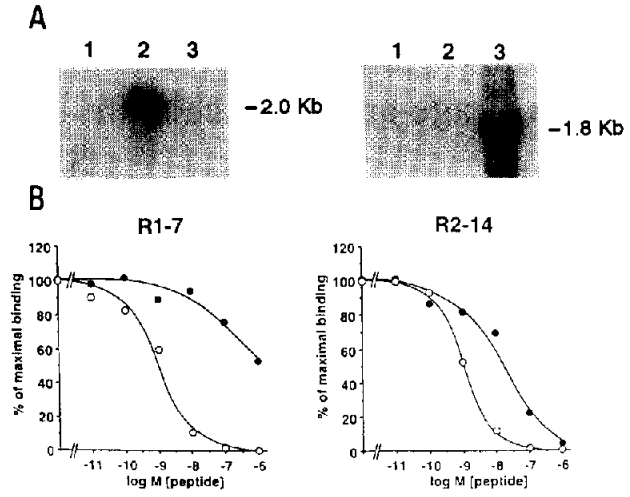

Fig. 1. (A) Northern blot analyses of hSSTR1 and 2 mRNAs in transfected RINm5F cells. Total cellular RNAs extracted from RINm5F cells transfected with expression plasmids carrying vector alone (lane 1), hSSTR1 gene (clone R1–7, lane 2), and hSSTR2 gene (clone R2–14, lane 3), were electrophoresed on 1% agarose gel, blotted to nylon membranes, and hybridized with ^{32}P-labeled hSSTR1 (left panel) and hSSTR2 (right panel) gene probes. The sizes of the hybridizing transcripts are indicated. (B) Binding of [^{125}I-Tyr^{11}]somatostatin-14 to RINm5F cells expressing hSSTR1 (R1–7) and hSSTR2 (R2–14). Data (the mean of triplicate determinations of 2 separate experiments) have been normalized to account for radiolabeled ligand bound in the absence of inhibitor (control binding, 1715 cpm for R1–7 and 1829 cpm for R2–14) and have been corrected for nonspecific binding (radio labeled ligand bound in the presence of 1 μM somatostatin, 518 cpm and 443 cpm in R1–7 and R2–14, respectively). Inhibition of binding in the presence of somatostatin (○) and SMS 201–995 (●) is shown.

3.4. Effects of GTPγS and PTX on somatostatin- and SMS 201-995-induced inhibition of VDCC currents in hSSTR2-expressing RINm5F cells

To further investigate the mechanism by which somatostatin and SMS 201-995 inhibit the HVA Ca^{2+} currents in R2–14 cells,

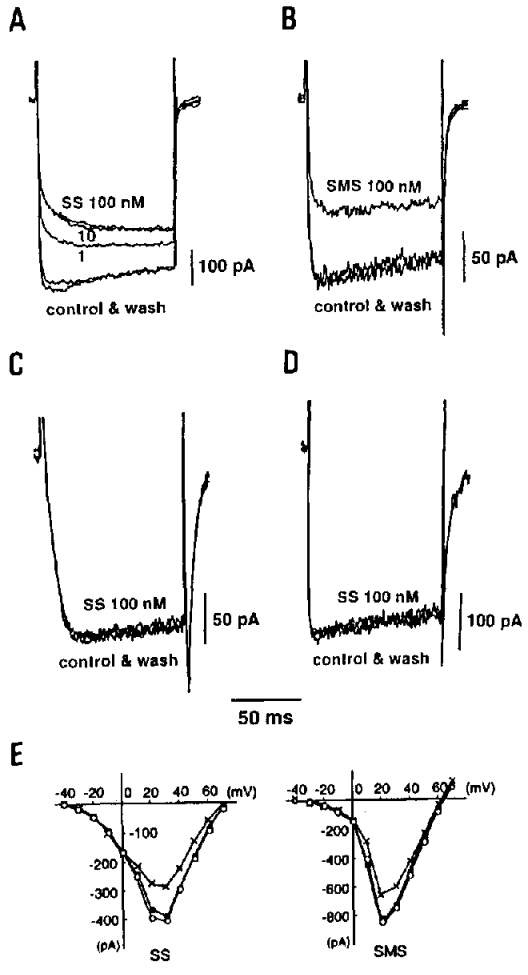

Fig. 2. Effects of somatostatin (SS) and SMS 201-995 (SMS) on VDCC currents in transfected RINm5F cells. Representative examples of VDCC current recordings in single RINm5F cells are shown. (A and B) R2–14 cells; (C) R1–7 cell; (D) RINm5F cell transfected with plasmid vector alone. Control and wash indicate before and after application of test materials, respectively. (E) Current–voltage relationships before (○), during (×), and after (●) exposure to somatostatin (SS) and SMS 201-995 (SMS) in R2–14 cells. Test pulse potential was +20 mV in A–D.

we have used a non-hydrolysable GTP analogue, GTPγS and PTX to characterize the properties of the G protein that couples SSTR2 to VDCC. When GTPγS was added to the pipette solution, the activation process of the HVA Ca^{2+} currents detected in RINm5F cells became progressively slower and the peak current amplitude was reduced to 72.2 ± 10.5% of control current (n = 5) in the first 10 min after breaking through the cell membrane with the patch pipette. The effect of GTPγS was evaluated 10–15 min after breaking through the cell membrane. As shown in Fig. 3B, the inhibitory effects of somatostatin and SMS 201-995 on the VDCC currents in R2–14 cells were relieved by the addition of GTPγS and also by the pretreatment of these cells with PTX.

3.5. *Effect of somatostatin on forskolin-induced cAMP production in the hSSTR1-expressing and hSSTR2-expressing RINm5F cells*

Since somatostatin is known to inhibit formation of cAMP through G_i signaling in many tissues [19], we examined the effect of somatostatin on forskolin-induced cAMP formation in R1–7 and R2–14 cells. Somatostatin inhibited forskolin-induced cAMP formation in both cells (Fig. 4) but it had no effect on forskolin-induced cAMP formation in control cells (data not shown), indicating that both hSSTR1 and hSSTR2 can be functionally coupled to adenylyl cyclase through an inhibitory G protein (G_i) in RINm5F cells.

4. Discussion

$[Ca^{2+}]_i$ plays important roles in many cellular functions [7,8]. Somatostatin exerts a variety of biological effects in neurons and endocrine cells by inhibition of Ca^{2+} influx through VDCC [9–12]. The diverse actions of somatostatin are mediated by its specific receptors. We recently have cloned five human somatostatin receptors (hSSTR1–5) [13–15]. In the present study we have found that somatostatin and its analogue SMS 201-995 inhibit VDCC currents detected in the rat insulinoma RINm5F cells stably transfected with hSSTR2 but not those transfected with hSSTR1. It has been reported that two types of VDCC currents are detected in pancreatic β-cells and various insulin-secreting clonal β-cells: a low voltage-activated (LVA) Ca^{2+} channel and a HVA Ca^{2+} channel [20]. Recent studies have indicated that ω-conotoxin sensitive HVA Ca^{2+} currents also are found in the RINm5F cells [21]. However, since we found that ω-conotoxin GVIA did not inhibit VDCC currents in RINm5F cells used in our experiments and also that the HVA Ca^{2+}currents were inhibited by somatostatin or SMS 201-995

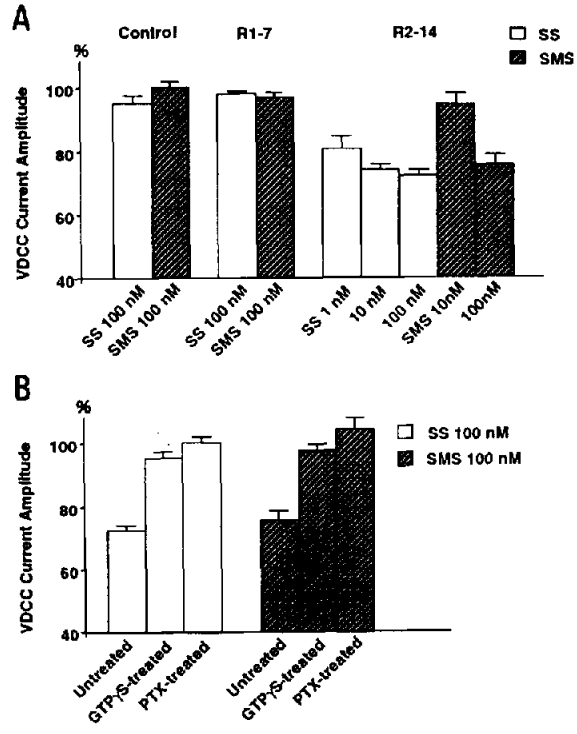

Fig. 3. (A) Summary of the effects of somatostatin (SS) and SMS 201-995 (SMS) on the VDCC currents in control (transfected with vector alone), R1–7, and R2–14 cells. The current amplitude obtained before the application of SS or SMS in each transfected cell is expressed as 100%. (B) Effects of GTPγS and PTX on VDCC current inhibition by somatostatin (SS) and SMS 201-995 (SMS) in R2–14 cells. The current amplitude obtained before the application of 100 nM SS or 100 nM SMS in R2–14 cell is expressed as 100%. Each value represents the mean ± S.E.M. of 5–18 experiments for A and 3–8 experiments for B.

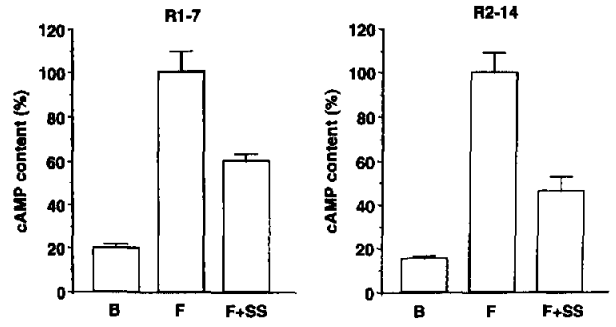

Fig. 4. Somatostatin (SS) inhibition of cAMP accumulation in RINm5F cells. In R1–7 cells, basal cAMP values (B) and forskolin-stimulated values (F) were 1.99 ± 0.16 pmol/well and 9.86 ± 0.99 pmol/well, respectively. In R2–14 cells, basal cAMP values (B) and forskolin-stimulated values (F) were 1.45 ± 0.13 pmol/well and 9.37 ± 0.89 pmol/well, respectively. The forskolin-stimulated cAMP values were used as control, and values are presented as percent of control (mean ± S.E.M., $n = 3$ in both cells).

after 10 min incubation with 10 μM ω-conotoxin GVIA (data not shown), the VDCC inhibited by somatostatin and SMS 201-995 in RINm5F cells are HVA Ca^{2+} channels other than N-type, probably L-type [20]. The reason why ω-conotoxin sensitive Ca^{2+} currents were not detected in RINm5F cells in the present study may be due to the different subline of RINm5F from that examined by G. Aicardi et al. [21]. Considering the fact that both hSSTR1 and hSSTR2 mediate the inhibition of cAMP accumulation and that hSSTR2 but not hSSTR1 mediates the inhibition of VDCC currents, it seems likely that a G protein other than G_i, most probably G_o, serves as a signal transducer for the inhibition of VDCC currents [22,23]. In fact, we have confirmed that both $G_{o\alpha1}$ and $G_{o\alpha2}$ are present in RINm5F cells, using reverse transcriptase-polymerase chain reaction (data not shown). $G_{\beta\gamma}$ also may participate in the inhibition of HVA Ca^{2+} currents by somatostatin and SMS 201-995 in RINm5F cells stably expressing hSSTR2, as has been reported of the somatostatin action on VDCC in rat pituitary GH_3 cells [24,25].

Although SMS 201-995 has been used clinically for the treatment of endocrine tumors such as pituitary adenomas, pancreatic tumors, and carcinoid, its mechanism of action is still unknown. We recently have determined the somatostatin receptor subtypes expressed in various endocrine tumors and have found that the efficacy of SMS 201-995 in inhibition of cell proliferation may depend in part on the expression of hSSTR2 [16]. Recent studies have suggested that somatostatin and SMS 201-995 may inhibit the cell proliferation process by activating phosphotyrosine phosphatase through G protein signal transduction [26,27]. The present study shows clearly that SMS 201-995 as well as somatostatin have their inhibitory effect on VDCC currents through SSTR2 which is coupled to a PTX-sensitive G protein, suggesting that the inhibition of calcium influx through VDCC may be one of the mechanisms by which SMS 201-995 inhibits cell proliferation and hormone secretion in endocrine tumors.

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