Selective stimulation of somatostatin receptor subtypes: differential effects on Ras/MAP kinase pathway and cell proliferation in human neuroblastoma cells

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Abstract In previous studies we have showed that somatostatin (SST) inhibits cell division, mitogen-activated protein (MAP) kinase and Ras activity in the human neuroblastoma cell line SY5Y. In the present study, we have assessed the role of a series of SST analogs, three of which were selective for SSTR1, SSTR2 or SSTR5, in these cellular events. All the analogs inhibited forskolin-induced cAMP accumulation. Selective stimulation of SSTR1 or SSTR2 but not of SSTR5 inhibited platelet-derived growth factor (PDGF)-induced [^{3}H]thymidine incorporation. The three analogs inhibited PDGF-stimulated MAP kinase activity, at least at an early time. In contrast, none of the analogs used individually was able to inhibit PDGF-stimulated Ras activity. A combined stimulation of SSTR2 and SSTR5 was necessary to obtain a significant inhibitory effect, suggesting the possibility of receptor heterodimerization. These results indicate that SST inhibition of Ras and MAP kinase activities takes place via different pathways and that SST inhibition of PDGF-induced cell proliferation occurs via a Ras-independent pathway.

Key words: Somatostatin receptor; Somatostatin analog; Human neuroblastoma; Signal transduction; Cell proliferation

1. Introduction

Somatostatin (SST) exerts a variety of physiological actions including inhibition of cell proliferation and hormone secretion via both indirect and direct mechanisms [1]. Interestingly, SST has also recently been suggested to possess antiangiogenic properties in rodents [2]. Based on experimental evidence from animals and cell cultures studies, the use of SST or the longer lasting analogs octeotride and lanreotide to suppress tumor cell proliferation in humans has been proposed [3]. However, clinical trials in cancer patients to date have not been very encouraging so that the therapeutic application of SST and the analogs is presently limited to the treatment of neuroendocrine tumors. Despite these negative results, study of the mechanisms underlying the antiproliferative action of SST might help to find new therapeutic approaches to cancer treatment. All the actions of SST are mediated by its interaction with specific membrane receptors of which five members have been cloned [4]. All the cloned SST receptors (SSTRs) belong to the family of seven transmembrane domain receptors, traditionally coupled to G proteins [4]. These receptors activate a variety of intracellular signals including inhibition of adenylate cyclase and Ca^{2+} channels and activation of K^+ channels and phosphatase activity. Pertussis toxin (PTx)-sensitive G proteins couple SSTR subtypes to their effectors [4]. Since the cloning of the five distinct SSTR subtypes, attempts have been made to attribute specific biological functions to each subtype. However, the lack of selective ligands has made it difficult to identify distinct functions for a particular receptor subtype. We have previously shown [5,6] that SST was capable of inhibiting cell proliferation, mitogen-activated protein (MAP) kinase and Ras activity in the human neuroblastoma cell line SY5Y. The very recent development of specific ligands for the SSTR1, SSTR2 and SSTR5 subtypes has allowed us to investigate the role of each of these receptors in the cellular processes affected by SST in SY5Y cells.

2. Materials and methods

2.1. Cell culture

The human neuroblastoma cell line SY5Y was grown in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum and kept at 37°C in a humidified atmosphere of 5% CO_2 in air.

2.2. RNA isolation and reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was isolated using a RNeasy total RNA isolation kit (Qiagen), following the supplier's protocol. cDNA was synthesized using M-MLV reverse transcriptase (Promega) with oligo(dT) primers and 10 ng of cDNA was subsequently used in the PCR reaction. Optimal temperature and cycling conditions were established for all five hSSTRs according to Panetta and Patel [7]. Amplified DNA fragments were visualized by 1.5% agarose gel electrophoresis. The primers used were described in [7]. Expected lengths for the amplified products were the following: SSTR1, 993 bp; SSTR2, 892 bp; SSTR3, 221 bp; SSTR4, 276 bp; SSTR5, 298 bp. The specificity of the amplified SSTR products was validated by sequencing (MWG-Biotech GmbH, Germany).

2.3. Intracellular cAMP assay

Cells were plated in 24-well dishes at a density of 2.5×10^5 cells/well. Before stimulation for 20 min with 50 μM forskolin in the presence of the analogs, the cells were incubated for 5 min with 1 mM isobutylmethylxanthine. Intracellular cAMP levels were quan-

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tified by radioimmunoassay using a commercial kit (Amersham Pharmacia Biotech, UK) after 95% ethanol extraction.

2.4. Proliferation assay

Quiescent SY5Y cells, plated at a density of 2×10^4 cells/well in 96-well dishes, were stimulated for 24 h with 20 ng/ml platelet-derived growth factor (PDGF) in the presence of the analogs. [^{3}H]Thymidine (1 μCi/well, specific activity 2 Ci/mmol, Amersham Pharmacia Biotech, UK) was added during the last 6 h of incubation. The cells were extracted in 10% trichloroacetic acid (TCA) and the radioactivity incorporated into TCA-insoluble material was evaluated after solubilization in 0.5 M NaOH.

2.5. MAP kinase activity assay

Quiescent SY5Y cells, plated in 60-mm Petri dishes, were washed with phosphate-buffered saline and lysed in 600 μl of lysis buffer as previously described [6]. MAP kinase was then immunoprecipitated from the lysates using a polyclonal anti-ERK2 antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and protein A-Sepharose beads. Immunocomplex kinase assay was performed as described in [6] using 250 μg/ml myelin basic protein (MBP, Gibco BRL, Grand Island, NY, USA) and [γ-^{32}P]ATP (3.0 μCi/sample; specific activity 3000 Ci/mmol; Amersham Pharmacia Biotech, UK) as substrates. An aliquot of the supernatants was transferred onto P81 phosphocellulose paper squares and after five washes in 0.75% phosphoric acid the radioactivity associated with the squares was determined by liquid scintillation counting.

2.6. Ras activity assay

GST-RBD (aa 51–131 of Raf-1) fusion protein was isolated in our laboratory from pGEX 2T-RBD induced with IPTG as previously described [8]. Activated Ras-GTP was precipitated from whole cell lysates prepared in lysis buffer (containing 50 mM HEPES–NaOH pH 7.4, 250 mM NaCl, 5 mM $MgCl_2$, 10% glycerol, 0.5 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin and 10 μg/ml leupeptin) with 20 μl glutathione agarose beads coupled with GST fusion protein containing the Ras binding domain (RBD) of Raf. After 12% SDS–PAGE, active Ras protein was detected by Western blotting using monoclonal anti-Ras antibodies (Transduction Laboratories, Lexington, KY, USA).

3. Results

3.1. SSTR subtype expression in SY5Y cells

SSTR subtype mRNAs were detected in SY5Y cells by RT-PCR using specific primers [7] (Fig. 1). The size of each PCR product corresponded to the predicted length of the cDNA fragment (993 bp, SSTR1; 892 bp, SSTR2; 221 bp, SSTR3; 276 bp, SSTR4; 298 bp, SSTR5). Confirmation of the identity to the SSTR sequences was obtained by sequencing all the PCR products (data not shown). As can be seen in Fig. 1, we observed the expression of all five SSTR subtypes in SY5Y cells.

3.2. SST analogs

SST binds to the five receptor subtypes with high affinity and little selectivity. A large number of analogs have been synthesized in the attempt to develop specific compounds with more selective actions and greater metabolic stability than the native hormone.

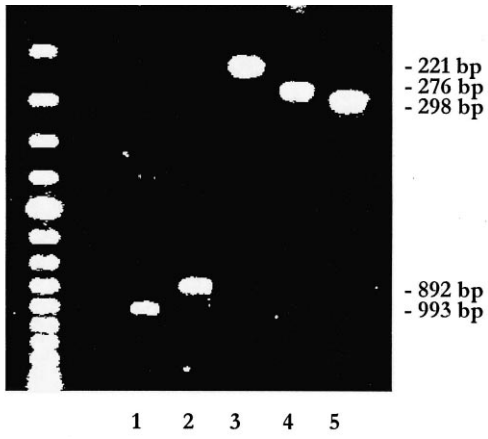


Fig. 1. SSTR subtypes expressed in SY5Y cells. RT-PCR was performed as described in Section 2. The size of each PCR product is shown on the right side of the photograph.

SST analogs Bim 23014, Bim 23120, Bim 23190, Bim 23197, Bim 23206, Bim 23268 and Bim 23745 were synthesized and supplied by Biomeasure, Inc. (Milford, MA, USA).

Receptor binding assays were performed using membrane preparations obtained from transfected CHO-K1 cells expressing the human SSTR subtypes 1–5 as described previously [9]. The IC_{50} values for the compounds used in our experiments are shown in Table 1. As already known, Bim 23014 (lanreotide/somatuline introduced into clinical use several years ago) binds preferentially to SSTR2 with moderate SSTR5 activity. Bim 23190 and Bim 23197 are more potent at SSTR2 with selectivity comparable to Bim 23014. Bim 23268 has high affinity for SSTR5 with some activity at all the other subtypes. Bim 23745, Bim 23120 and Bim 23206 are fully selective analogs for the SSTR1, 2 and 5 subtypes respectively, as can be seen from the IC_{50}s presented in Table 1. This is the first report showing the binding affinities for these three analogs and the method used for determining the K_i is as reported in [9]. In our experiments these three compounds were used as specific ligands for the individual SST receptor subtypes 1, 2 and 5. There are still no SSTR3- and SSTR4-selective agonists.

3.3. SST analogs and cAMP formation

All five receptor subtypes couple in an inhibitory fashion to adenylate cyclase via a PTx-sensitive G protein [4]. Fig. 2

Table 1
Binding affinity of SST and its analogs for the cloned hSSTR subtypes

	IC_{50} (nM)				
	SSTR1	SSTR2	SSTR3	SSTR4	SSTR5
Bim 23745	***42***	>1000	>1000	>1000	1480
Bim 23120	1000	***0.34***	412	1000	213.5
Bim 23206	1152	166	1000	1618	***2.4***
Bim 23190	5210	***0.35***	215	7537	***11.2***
Bim 23197	6016	***0.19***	26.8	3897	***9.8***
Bim 23268	***12***	***28***	***5.5***	***36***	***0.42***
Bim 23014	2129	***0.75***	98	1826	***12.7***
SST-14	***1.95***	***0.25***	***1.2***	***1.77***	***1.41***

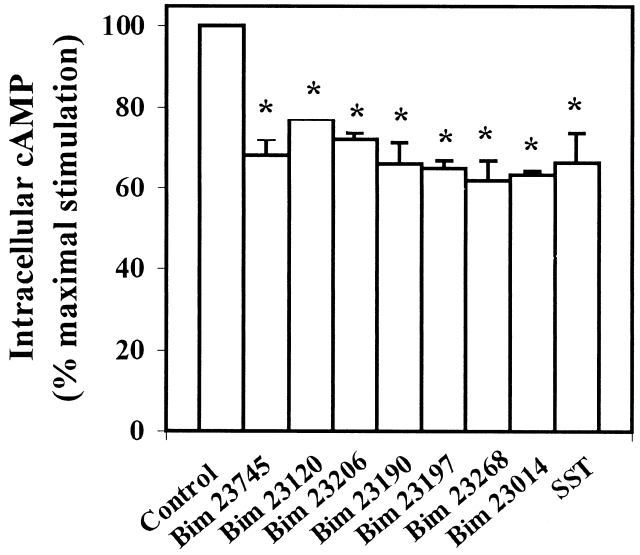


Fig. 2. Effect of SST and its analogs on intracellular cAMP levels. SY5Y cells were treated for 20 min with 50 μM forskolin in the absence or in the presence of the following drugs: Bim 23745 (200 nM), Bim 23120 (20 nM), Bim 23206 (20 nM), Bim 23190 (20 nM), Bim 23197 (100 nM), Bim 23268 (200 nM), Bim 23014 (20 nM) and SST (10 nM). The intracellular cAMP levels were assayed by radioimmunoassay after ethanol extraction. Data are expressed as percent maximal stimulation observed with forskolin alone. Each histogram is the mean ± S.E.M. of three independent experiments, each performed in duplicate. $^{*}P < 0.005$, Student's *t*-test.

shows that all the analogs tested inhibited forskolin-stimulated cAMP accumulation in SY5Y cells. Maximal accumulation of cAMP by 50 μM forskolin, set at 100%, was reduced to 66% after pretreatment with SST, i.e. the inhibition induced by the native hormone was 34%. The inhibition of cAMP accumulation by the analogs was 32%, 22% and 28% for Bim 23745 (200 nM), Bim 23120 (20 nM) and Bim 23206 (20 nM), respectively. The inhibition by the other non-selective analogs ranged between 34 and 36%. These results imply that: (1) SST receptors expressed in SY5Y cells are functionally active; (2) all the analogs tested behave as SSTR agonists.

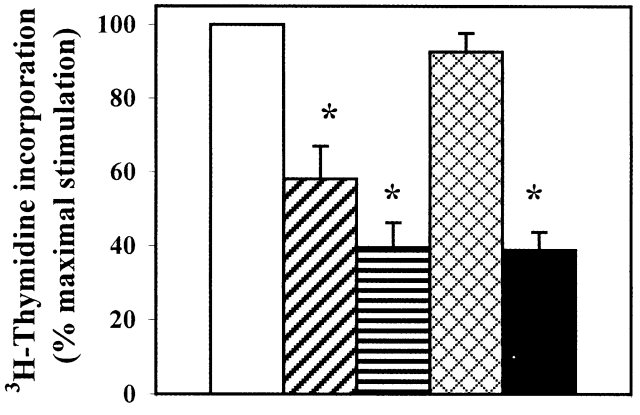


Fig. 3. Effect of SST and its analogs on PDGF-induced DNA synthesis. Quiescent SY5Y cells were stimulated with 20 ng/ml PDGF in the absence (open bar) or in the presence of Bim 23745 (200 nM, diagonal bar), Bim 23120 (20 nM, horizontal bar), Bim 23206 (20 nM, cross-hatched bar) or SST (10 nM, solid bar). [^{3}H]Thymidine incorporation by the cells was evaluated as described in Section 2. Results are expressed as percent maximal stimulation induced by PDGF and are the mean ± S.E.M. of 3–5 independent experiments, each performed in triplicate. $^{*}P < 0.005$, Student's *t*-test.

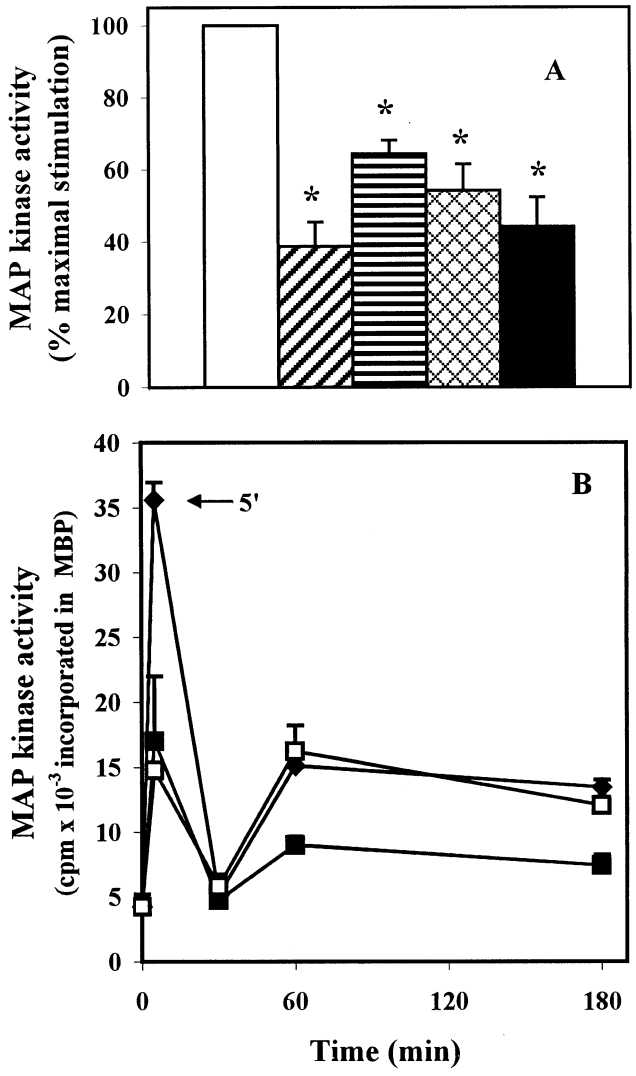


Fig. 4. Effect of SST and its analogs on PDGF-induced MAP kinase activity. A: Quiescent SY5Y cells were treated for 8 min with Bim 23745 (200 nM, diagonal bar), Bim 23120 (20 nM, horizontal bar), Bim 23206 (20 nM, cross-hatched bar) or SST (10 nM, solid bar) before stimulation for 5 min with 20 ng/ml PDGF (open bar: no Bim). MAP kinase activity was evaluated by immunocomplex kinase assay as described in Section 2. Results are expressed as percent maximal stimulation induced by PDGF and are the mean ± S.E.M. of four independent experiments, each performed in duplicate. $^{*}P < 0.005$, Student's *t*-test. B: Cells were treated with PDGF 20 ng/ml for the indicated times in the absence (♦) or in the presence of SST 10 nM (■) or Bim 23206 20 nM (□). MAP kinase activity was evaluated as in A. Results are expressed as cpm of ^{32}P incorporated in MBP and are the mean ± S.D. of a representative experiment which was repeated once with similar results.

3.4. SST analogs and cell proliferation

To analyze the specific biological function coupled to SSTR subtypes, SY5Y cell proliferation was tested in the presence of SSTR1, 2 or 5 agonists (Fig. 3). Bim 23120 (20 nM) inhibited PDGF-stimulated [^{3}H]thymidine incorporation to the same extent (60%) as SST. Bim 23745 (200 nM) was a little less efficient (42% inhibition), while Bim 23206 (20 nM) did not

affect [^{3}H]thymidine incorporation at all. These data indicate that, while the receptor subtypes 1 and 2 individually stimulated are capable of inhibiting cell proliferation stimulated by PDGF, subtype 5 is ineffective.

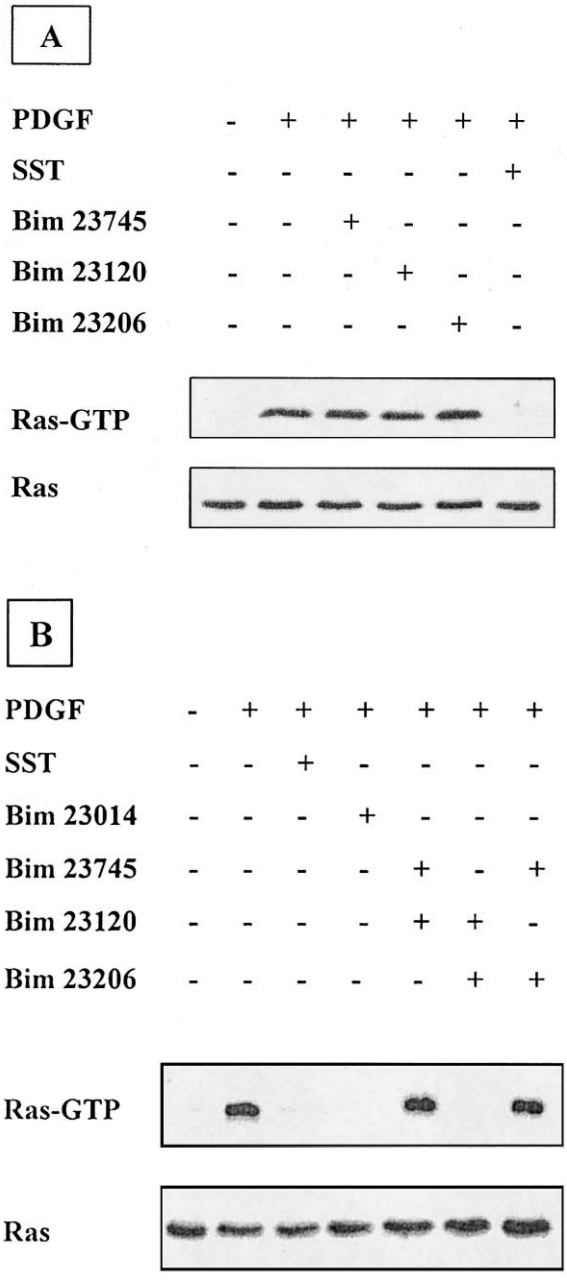


Fig. 5. Effect of SST and its analogs on PDGF-induced Ras activity. A: Serum-deprived SY5Y cells were pretreated for 8 min with Bim 23745 (200 nM), Bim 23120 (20 nM), Bim 23206 (20 nM) or SST (10 nM) before stimulation with PDGF (20 ng/ml) for 4 min. Ras was identified by affinity precipitation with GST-RBD followed by immunoblotting (upper panel) and by immunoblotting of total cell lysates (lower panel) using anti-Ras antibodies. B: The experiment was performed as in A, but the analogs at the concentrations indicated above were added simultaneously in the following combination: Bim 23745+Bim 23120, lane 5; Bim 23120+Bim 23206, lane 6; Bim 23745+Bim 23206, lane 7. SST (lane 3) and Bim 23014 (lane 4) were added at concentrations of 10 nM and 20 nM, respectively. Ras was identified as described in A. Each type of experiment was repeated three times with similar results.

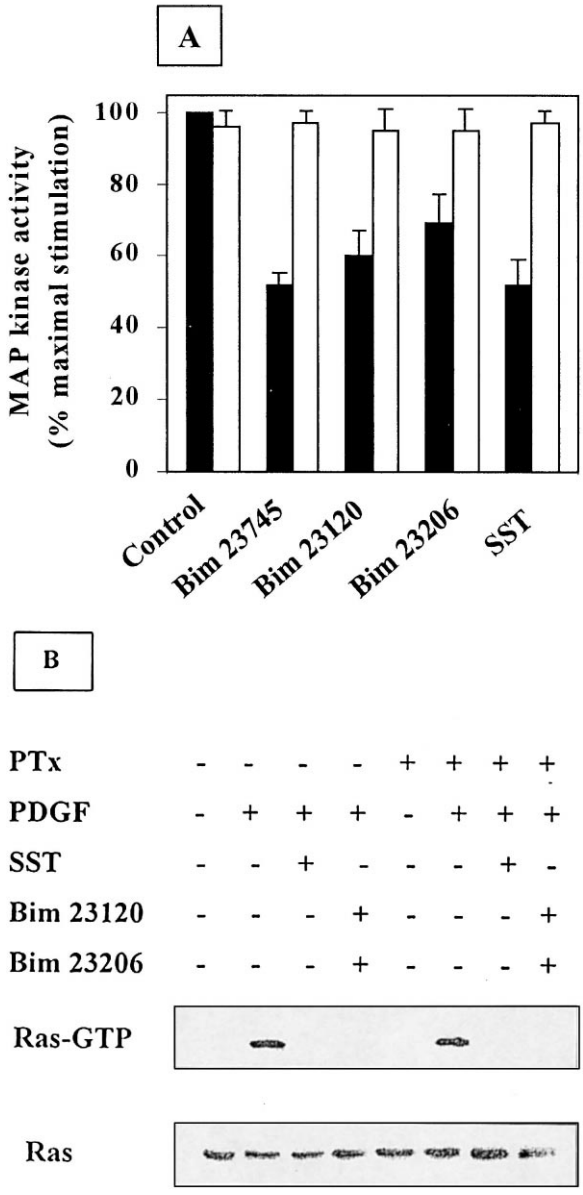
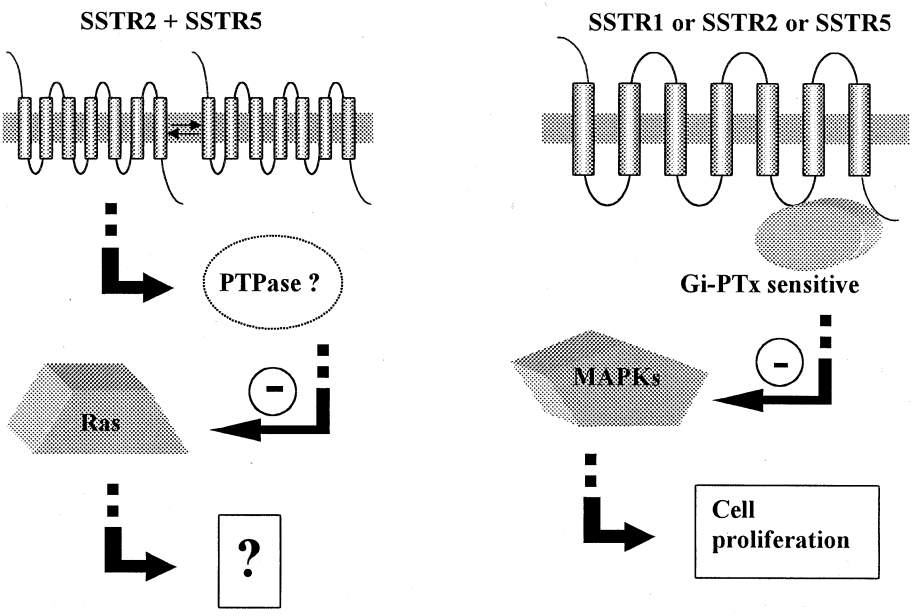


Fig. 6. Differential effect of PTx treatment on SST- and analog-mediated inhibition of MAP kinase and Ras activity both induced by PDGF. A: Serum-deprived SY5Y cells were incubated for 4 h in the presence (open bars) or in the absence (solid bars) of 100 ng/ml PTx. The experiments and the evaluation of the MAP kinase activity were then performed as described in Fig. 4. Results are expressed as percent maximal stimulation induced by PDGF and are the mean ± S.E.M. of three independent experiments, each performed in duplicate. B: Serum-deprived SY5Y cells, preincubated where indicated with PTx as in A, were treated with 10 nM SST or with the combination 20 nM Bim 23120+20 nM Bim 23206 before stimulation with 20 ng/ml PDGF for 4 min. Ras activity was measured as described in Fig. 5. This experiment was repeated three times with similar results.

3.5. SST analogs and MAP kinase activity

MAP kinase as well as Ras activities are considered crucial events in growth factor signalling [10]. We have previously reported that SST inhibited serum- and PDGF-stimulated MAP kinase activity in SY5Y cells [5,6]. Fig. 4A shows that the analogs specific for the receptor subtypes 1, 2 and 5 inhibited the PDGF-stimulated MAP kinase activity by 61, 36 and 48%, respectively. For this assay, overnight serum-de-



Scheme 1. Schematic representation of the hypothetical mechanisms suggested for the inhibitory effect of SST on Ras and MAP kinase activities.

prived cells were preincubated for 8 min with SST (10 nM) or with the three analogs individually at the concentrations previously indicated, and then stimulated with PDGF (20 ng/ml) for 5 min. MAP kinase activity was measured by immunocomplex kinase assay as described in Section 2.

Also in Western blot experiments using antibodies against the phosphorylated forms of ERK-1 and ERK-2 the analogs decreased the phosphorylation levels of both the enzymes (data not shown).

We have previously described that the analog specific for SSTR5 did not affect cell proliferation (Fig. 3) and yet here it does inhibit MAP kinase activity. Since it has been suggested that a prolonged activation of MAP kinase is needed for stimulation of cell proliferation [11], we measured the effect of Bim 23206 after a longer time of PDGF stimulation. Fig. 4B shows the typical biphasic kinetics of MAP kinase activity stimulated by growth factors (in our case PDGF) [11]. It can be seen that while SST was capable of inhibiting MAP kinase activity even after a prolonged (1 and 3 h) stimulation with PDGF, the analog did not and at these later times MAP kinase activity was back to control levels. Thus, it is possible to speculate that the lack of effect of Bim 23206 on cell proliferation could be ascribed to its inability to induce a long-lasting MAP kinase inhibition.

3.6. SST analogs and Ras activity

We have previously shown [6] that SST inhibited the PDGF-stimulated Ras activity by an assay of cell lysate immunoprecipitation with GST-RBD (the minimal Ras binding domain of Raf) which binds to the active form of Ras. Fig. 5A shows that the single analogs were not capable of inhibiting PDGF-stimulated Ras activity. Only the combined action of the analogs specific for SSTR2 and SSTR5 (but not of those specific for SSTR1 and 2 or SSTR1 and 5) induced a dramatic decrease of the active Ras (Fig. 5B). Moreover, the analog Bim 23014, which displays high affinity for SSTR2 and moderate activity for SSTR5, depressed Ras activity. The analog Bim 23197, which possesses a profile similar to Bim 23014, behaved in the same way (data not shown). Thus, the simultaneous stimulation of SST receptor subtypes 2 and 5 is needed to obtain the inhibitory effect on Ras. Equal amounts of total Ras proteins were subjected to the RBD binding assay as shown by immunoblotting of an aliquot of total cell lysate followed by detection with anti-Ras antibodies (lower panel).

Our results indicate that SST may inhibit MAP kinase and Ras activity via two different mechanisms (Scheme 1). This conclusion is further supported by the experiments presented in Fig. 6. SY5Y cells either were or were not pretreated with PTx for 4 h and then assayed for MAP kinase (Fig. 6A) and Ras (Fig. 6B) activity stimulated by PDGF in the presence or in the absence of SST or the analogs. It can be seen that while the inhibitory action of SST and SST analogs on MAP kinase is abolished by PTx, Ras activity is still inhibited by the hormone and analogs even in the presence of the toxin.

4. Discussion

Five distinct SSTR subtypes have been cloned and often more than one subtype is expressed in the same target cell; therefore it is reasonable to assume that each SSTR subtype could serve distinct biological functions and couple to distinct effectors via specific mechanisms. It is also reasonable to think that there could be additional functions modulated by the combined action of two or more SSTR subtypes.

Two factors allowed us to begin the difficult task of defining specific roles for SSTR subtypes. First, three selective ligands for SSTR1, 2 and 5 were made available to us. Second, we had at our disposal in the laboratory a human neuroblastoma cell line SY5Y where SST suppresses cell proliferation and in which all five SSTR subtypes are expressed, according to our RT-PCR studies. With these tools it was possible to begin to at least partially unravel which SSTR subtype(s) is responsible for the SST antiproliferative effect and which signalling mechanisms are involved.

Up to now, the role of a receptor subtype on cell responses has been studied in cells transfected with individual SSTR subtypes where the high number of receptors expressed may favor a less selective coupling to the effectors. In our case we are measuring the response of the native receptors in their natural environment. The receptor subtypes considered are functional since they respond to the application of the analogs by decreasing forskolin-stimulated cAMP accumulation. Regarding the antiproliferative action, we have shown that the stimulation of SSTR1 and SSTR2 individually, but not of SSTR5, is sufficient to inhibit cell proliferation stimulated by PDGF. SSTR2 stimulation was as effective as SST itself, while the SSTR1 ligand induced a slightly smaller inhibition. The lack of effect of the SSTR5 ligand as antiproliferative agent in this case could be ascribed to its inability to induce a long-lasting inhibition of MAP kinase activity stimulated by PDGF. It has in fact been suggested that the prolonged phase of MAP kinase activation by growth factors is needed to allow the complex cellular machinery for cell division to set in motion [11]. SSTR5 was able to inhibit only the early phase of enzyme activation, allowing the late phase to occur unencumbered. From the literature, it appears that there are differences in the cellular regulation of the five SSTRs and a different pattern of internalization has been described for each of them [12,13]. One can hypothesize that in our cells SSTR5 receptors may undergo a desensitization and internalization faster than the other two subtypes thus preventing a long-term action.

In our opinion, one of the most interesting findings of this report is the need for a combined action of SSTR2 and 5 (and not of SSTR1 and 2 or SSTR1 and 5) to inhibit Ras activity. The formation of dimers for tyrosine kinase receptors is well assessed but it has only recently been proposed for G protein-coupled receptors (for a review and a comment see [14,15]). Recent evidence that some members of the SSTRs family undergo heterodimerization [16] is in line with our result. The dimeric association has been shown to alter some functional properties of the receptors such as binding affinity and internalization [16]. This report and our present results suggest that multiple SSTR subtypes expressed in the same cells are not simply redundant but interact functionally and specifically with each other (in our hands only SSTR2 and 5 combination inhibits Ras activity) to provide greater signalling diversity.

A further interpretation of our results is that MAP kinase inhibition occurring after individual SSTR stimulation is mediated by a different pathway than Ras inhibition. This conclusion is confirmed by the different sensitivity to PTx displayed by the analogs on MAP kinase and Ras activity inhibition. We had previously reported that SST inhibition of Ras activity is insensitive to PTx treatment and suggested that either a PTx-insensitive G protein is associated with SSTR or that there is a direct association of the SSTR with the effector bypassing the requirement of a G protein [6]. This putative G protein-independent SSTR–effector interaction might involve a PDZ domain. The consensus sequence for binding PDZ domains (T/SXV/L/I) is present on the C-terminal tail of both SSTR2 and 5 subtypes. An interaction between a PDZ bearing protein (CortBP1) and the rat SSTR2 subtype has been recently reported in transfected human embryonic kidney cells [17]. Experiments to further elucidate these mechanisms are under way in our laboratory. The SST inhibition of MAP kinase activity is, in contrast, PTx-sensitive and involves the interaction of a PTx-sensitive G protein with the effector. From our results it also derives that Ras activity is not correlated with MAP kinase activity nor with cell proliferation in SY5Y cells.

In conclusion, the data presented here suggest that the action of SST is very complex. Its interaction with the specific receptors and possibly with receptor heterodimers produces a variety of intracellular signals via diverse mechanisms which may very well explain the multiplicity of SST effects on target cells. Due to this complexity, it is important to identify specific roles for the receptor subtypes. Further studies with selective analogs will lead to a better understanding of SSTR functions and to the discovery of compounds able to target specific physiological effects and pathological conditions.

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