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Somatostatin Analog SMS 201995 Inhibits Proliferation in Human Leukemia T-Cell Line: Relevance of the Adenylyl Cyclase Stimulation

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Abstract Octreotide SMS 201995 is a stable somatostatin (SRIF) analog with potent antiproliferative actions in numerous cell types including normal T lymphocytes. It is currently used in the clinical treatment of different malignancies. However, the possible beneficial actions of octreotide in T-cell leukemia have not been addressed before, although these cells express SRIF receptors. For instance, human leukemia Jurkat T cells have been shown to express a single SRIF receptor isotype: sst3 that can be pharmacologically targeted by octreotide. In this study, we therefore studied SMS 201995 effects on in vitro [^{3}H-CH3]thymidine incorporation in Jurkat T cells. Our data show that octreotide inhibits the proliferation of Jurkat cells both in the absence and in the presence of mitogens. By contrast, SRIF28, an endogenous SRIF analog sharing with SMS 201995 an almost identical affinity for somatostatin sst3 receptors, increases [^{3}H-CH3]thymidine uptake in both mitogen-activated and nonactivated cells. To assess the mechanisms of the opposite actions of these two analogs on leukemia T-cell proliferation, we next studied their effects on adenylyl cyclase activity in whole Jurkat cells. At least in the presence of mitogens, SMS 201995 significantly enhances the adenylyl cyclase activity whereas SRIF28 inhibits it. Taken together these data are in accordance with the current hypothesis according to which increase and decrease in cAMP production are required to allow the inhibition and stimulation of T-cell proliferation, respectively. They also point to a potential therapeutic benefit of SMS 201995 in the management of human T-cell leukemia. J. Cell. Biochem. 78:666–673, 2000.

Key words: somatostatin; proliferation; mitogenic activation; Jurkat T cells; adenylyl cyclase

Activation of T cells in vivo by antigen-presenting cells is a result of the engagement of both the T-cell receptor–CD3 (TCR-CD3) complex and the CD28 costimulatory receptor. Simultaneous binding of appropriate ligands to both receptors is required for triggering interleukin-2 (IL-2) production and proliferation in a process generally called T-cell activation [Janeway and Bottomly, 1994], which can be mimicked in vitro by mitogenic stimulation. T-cell activation is disregulated in pathologies of T-cell proliferation: leukemia cells proliferate even in the absence of IL-2 [Taniguchi and Minami, 1993].

Transduction pathways engaged in TCR-CD3 signaling involve the activation of nonreceptor tyrosine kinases during the initial phase of T-cell response [Tamir and Cambier, 1998]. CD28 stimulation leads to an activation of phosphodiesterases (cAMP-degrading enzymes), resulting in a decrease of the intracellular cAMP concentration and, given that cAMP is the sole physiologic regulator of PKA activity, subsequent inhibition of protein kinase A (PKA) [Li et al., 1999]. It has been recently proposed that the constitutive activity of cAMP-PKA pathway exerts a "tonic" inhibition of T-cell proliferation and that the full activation of T cells requires additional TCR- and CD28-independent signals to inhibit PKA and overcome PKA-related inhibition of proliferation [Tamir et al., 1996]. The mechanisms of

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PKA-related inhibition of T-cell proliferation have been elucidated: They impinge directly on the signals necessary for IL-2 gene expression [Hsueh and Lai, 1995; Rao et al., 1997; Tsuruta et al., 1998]. As a consequence, the T-cell activation process is disrupted by agonists that stimulate the cAMP-PKA pathway [Tamir et al., 1996]. However, the impact of agonist-driven inhibition of the cAMP-PKA pathway is less known.

The wide-spread 14-amino-acid-composed isoform of somatostatin (SRIF14) and its naturally occurring counterpart composed of 28 amino acids (SRIF28) inhibit cAMP-PKA pathway in all mammalian cells studied, including T cells. The underlying mechanisms implicate a direct inhibition of adenylyl cyclase (cAMP-producing enzyme) and subsequent decrease of the intracellular cAMP concentration. These SRIF actions are mediated by the five (sst1–sst5) known somatostatin receptors all coupled to the enzyme via Giα protein [Reisine and Bell, 1995]. In agreement, the modulatory actions of SRIF14 on T-cell proliferation and IL-2 production are well recognized [Van Hagen et al., 1994]. Nevertheless, SRIF14 not only stimulates the proliferation of T cells [Nordlind and Mutt, 1985; Nordlind and Mutt, 1986; Johansson and Sandberg, 1989] as it would be expected based on its capacity to inhibit cAMP-PKA pathway [Kammer, 1988], but is also able to inhibit it [Payan et al., 1984; Pawlikowski et al., 1985; Nio et al., 1993]. Similarly, in other cell types, both SRIF14-dependent positive [Johansson and Madsen, 1987] and negative [Tsuzaki and Moses, 1990; Kokudo et al., 1991] regulation of proliferation has been documented. By contrast, a metabolically stable SRIF14 analog, octreotide SMS 201995, inhibits cell proliferation in almost all cell types studied [Feindt et al., 1997; Pawlikowski et al., 1997a; Pawlikowski et al., 1997b; Yumi et al., 1997; Feindt et al., 1998] including normal human T lymphocytes [Malec et al., 1989; Atiya et al., 1997]. The absence of significant inhibitory actions of octreotide on proliferation has been reported by only a few studies [Liebow et al., 1989; Alper et al., 1997; Bodenant et al., 1997], whereas an increase of proliferation has never been observed with this analog.

SMS 201995 displays sst2, sst3, and sst5 receptor selectivity on human somatostatin receptors [Patel and Srikant, 1994] and is currently used in the clinical treatment of different (neuro)endocrine and digestive tumors [Lamberts et al., 1991; Reubi, 1997]. However, possible beneficial actions of SMS 201995 in the management of T-cell leukemia have never been addressed, although various leukemia T cells express somatostatin receptors on their surfaces [Hiruma et al., 1990; Cardoso et al., 1998].

We recently reported that SMS 201995 modulates adenylyl cyclase via sst3 somatostatin receptor in plasma membrane preparations of human leukemia T-cell line, Jurkat [Giannetti et al., 1999]. In the present study, we developed a whole-cell adenylyl cyclase assay and attempted to correlate SMS 201995 actions on cAMP production by adenylyl cyclase on the one hand and proliferation on the other hand. Furthermore, we assessed the underlying mechanisms of SMS 201995 actions by comparing its effects on the two parameters studied (i.e., proliferation and adenylyl cyclase activity) with those of SRIF28 (the latter having a nearly identical affinity for human sst3 receptor as SMS 201995 [Patel and Srikant, 1994]).

MATERIALS AND METHODS

Chemicals

[^{3}H]adenine (26 Ci/mmol) was from Amersham (Amersham, UK). SRIF28 was obtained from Peninsula Laboratories (San Carlos, CA), and SMS 201995 was a kind gift from Novartis (Basel, Switzerland). All other chemicals were purchased from Sigma (L'Isle d'Abeau, France).

Cell Culture and Mitogen Stimulation

Jurkat cells (clone J77.6.8) were grown in RPMI 1640 medium (Gibco, Cergy-Pontoise, France) supplemented with 10% (weight/volume) heat inactivated fetal calf serum (FCS) (Boehringer, Meylan, France), 10 mM HEPES, 2 mM L-glutamine and 100 U/ml gentamycin in 5% CO_2 humidified atmosphere at 37°C.

Jurkat cell suspensions (0.5×10^6 cells/ml) were either left untreated (control group corresponding to nonactivated cells) or incubated with a combination of phorbol ester, 12-o-tetradecanoyl-13-phorbol acetate (TPA) (16 nM), and lectin phytohemagglutinin-leukocytes (PHA-L) (2 μg/ml, Sigma) for 18 h (mitogen-activated cell group). Only cell batches in which viability exceeded 90% (as determined by trypan blue exclusion test) were used for further experiments.

An aliquot of cell culture supernatant was assayed for IL-2 content (18 h after mitogen stimulation) by using an IL-2–enzyme-linked immunosorbent assay (ELISA) immunoassay kit (Immunotech, Marseille, France) according to the manufacturer's instructions. This served as an additional criterion of lymphocyte activation since resting T lymphocytes do not produce IL-2 [Taniguchi and Minami, 1993].

Cell Proliferation Assay

Activated and nonactivated cells were cultured in 96-well plaques in 200 μl final volume (2×10^5 cells per well) of the complete, 10% FCS-supplemented RPMI medium in the absence or in the presence of either SMS 201995 or SRIF28 (1 pM to 10 nM). [^{3}H-CH3]thymidine (Du Pont, NEN, Les Ulis, France) (specific activity: 88.7 Ci/mmol) was added at the rate 1 μCi per well at the same time as either SMS 201995 or SRIF28, and incubation was carried out for 5 h. The reaction was stopped by filtration on a cell harvester (Tomtec) and the filters were counted in a liquid scintillation beta counter (Wallac 1450 Microbeta Trilux).

Adenylyl Cyclase Assay in Whole Cells

Adenylyl cyclase activity was measured in whole cells after incorporation of [^{3}H]adenine to label the intracellular pool of ATP. The product (cAMP) and the substrate (ATP) of the enzymatic reaction were separated by chromatography on Dowex and alumina columns according to a previously described method [Salomon et al., 1973]. Briefly, Jurkat cells (10^6/ml) were incubated in RPMI 1640 (10% FCS; 10 mM HEPES; 2 mM L-glutamate, and 1/1000 gentamicine) supplemented with 1 mM of an inhibitor of phosphodiesterases isobutylmethylxanthine (IBMX) and [^{3}H]adenine (5μCi/ml) overnight. The radioactive supernatant was discarded and cells were washed twice. The cells were subsequently preincubated for 15 min as a suspension containing 2×10^6 cells per well (24-well plates, Costar, Cambridge, MA) in RPMI 1640 (0% FCS; 10 mM HEPES; 2 mM L-glutamate, 1/1000 gentamicine) containing 1 mM of IBMX and supplemented with 0.5 g/liter bacitracin to limit the proteolitical degradation of peptides. Somatostatin analog (1 pM to 10 nM) actions on the enzyme activity were studied over a 15-min incubation period. The cultures were then harvested, cells sedimented by centrifugation (1000*g*, 5 min, 25°C), and the cellular proteins denatured by adding ice-cold trichloracetic acid (5%) containing nonradioactive cAMP (1mM) and ATP (1mM) to the cell pellet. Radioactivity was measured by liquid scintillation counting. The results were expressed as the ratio of [^{3}H]cAMP recovered from the alumina eluate over [^{3}H]ATP recovered from the Dowex eluate fold 1000.

Data Analysis

Results were expressed as means ± SEM. The experiments were run in triplicate (cell proliferation) or duplicate (adenylyl cyclase) and repeated three or four times. Statistical analysis included the evaluation of experimental differences by analysis of variance (ANOVA) using an In Stat computer program (GraphPad Software, Inc., San Diego CA). Differences were considered significant for the *P*-value of F-statistics less than 0.05.

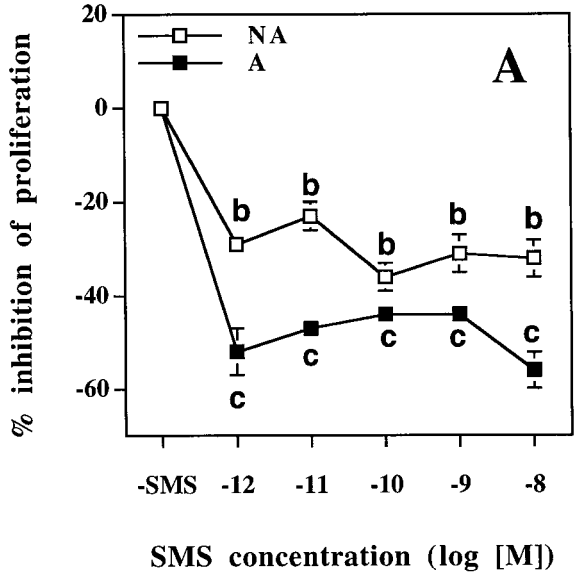
RESULTS

Modulation of Jurkat Cell Proliferation

We first ascertained that Jurkat cells can be properly stimulated by mitogens. No detectable IL-2 secretion could be evidenced in the supernatant of cells cultured without mitogens. By contrast, in the supernatant of mitogen-activated Jurkat cells, IL-2 content was 1.185 ± 0.003 ng/ml, thus indicating that activation-related induction of the growth factor secretion occurs in our experimental conditions.

We then studied the actions of octreotide (SMS 201995) on proliferation of Jurkat cells cultured in the absence or presence of mitogens. Given that Jurkat cells express only sst3 out of five cloned SRIF receptors, we chose to compare octreotide actions on proliferation to those of SRIF28. The latter was chosen as a reference because its affinity for human sst3 receptor is closer to the affinity of SMS 201995 than is the affinity of the generally studied somatostatin analog, SRIF14 [Patel and Srikant, 1994].

Analysis of variance indicates a significant ($F = 18.9$, $P < 0.0001$) SMS 201995-dependent inhibition of proliferation in nonactivated Jurkat cell. Indeed, in the absence of mitogens, SMS 201995 inhibits [^{3}H-CH3]thymidine incorporation with a maximum (−36%) being



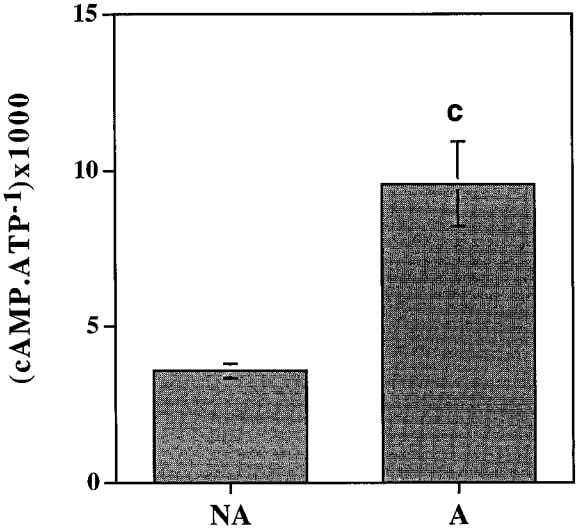


Fig. 2. Basal (i.e., in the absence of somatostatin analogs) cAMP production in nonactivated (NA) and mitogen-activated (A) Jurkat cells. Each bar corresponds to the mean ± SEM obtained from duplicates in four different experiments (n = 8). c, $P < 0.001$.

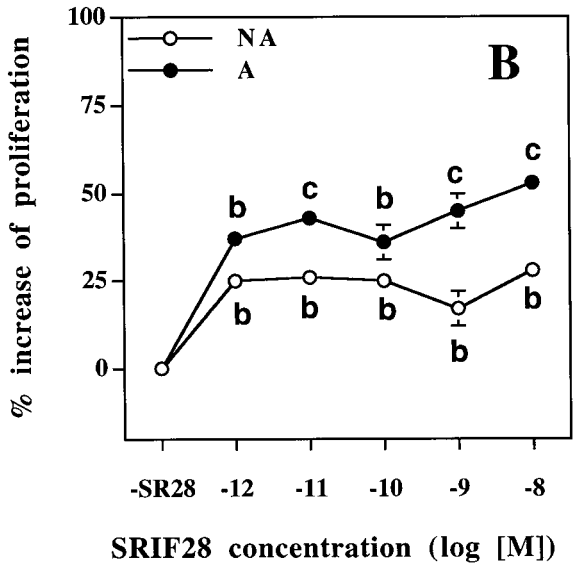


Fig. 1. SMS 201995 (**A**) and SRIF28 (**B**) actions on proliferation of nonactivated (open symbols, NA) and mitogen-activated (filled symbols, A) Jurkat cells. Data are expressed as percentage of [^{3}H-CH3]thymidine incorporation measured in the absence of the analogs taken as a reference 100%. This reference value corresponds to 45420 ± 3560 cpm (A) and 53007 ± 1581 cpm (B). Depicted points represent mean ± SEM from triplicate determinations obtained in three independent experiments (n = 9). b, $P < 0.01$; c, $P < 0.001$.

reached at 0.1 nM analog concentration (Fig. 1A).

In mitogen-activated Jurkat cells, SMS 201995 also inhibits (F = 56.4, $P < 0.0001$) cell proliferation. The observed inhibition varies between −50 and −60% and was measured in the whole concentration range (1 pM to 10 nM) studied (Fig. 1A).

In contrast, SRIF28 significantly (F = 19.2, $P < 0.0001$) increases [^{3}H-CH3]thymidine uptake in nonactivated cells. This SRIF28-mediated enhancement of proliferation is even more pronounced (F = 33.7, $P < 0.0001$) in mitogen-activated cells. The maximal stimulation is measured for the peptide concentrations greater than 0.1 nM. In this concentration range, the stimulation equals +40% to +60% of [^{3}H-CH3]thymidine incorporation determined in the absence of SRIF28 (Fig. 1B).

Modulation of Adenylyl Cyclase Activity

To determine if the mechanism of the observed opposite effects of SMS 201995 and SRIF28 on cell proliferation could be related to their possible different actions on adenylyl cyclase activity, we next measured the enzyme activity in whole nonactivated and activated Jurkat cells. During the setup of experimental conditions it was noticed that the addition of mitogens per se (i.e., in the absence of somatostatin analogs) increases basal adenylyl cyclase significantly (F = 18.9, $P < 0.0005$) when compared to that measured in nonactivated cells (Fig. 2).

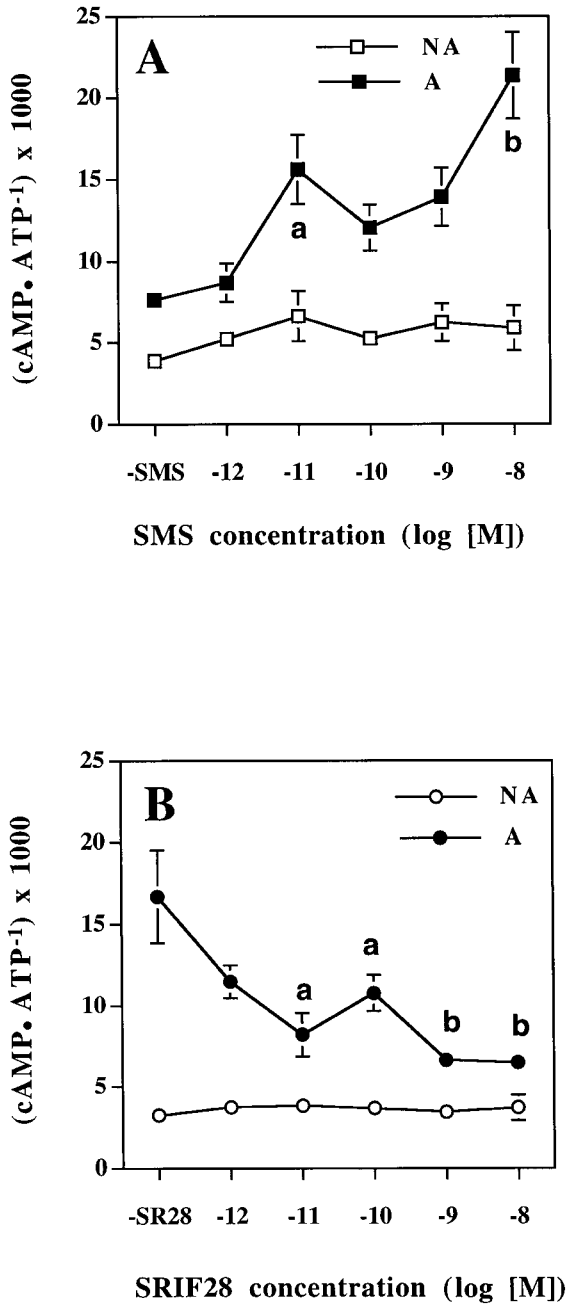


Fig. 3. Effects of increasing concentrations of SMS 201995 (**A**) and SRIF28 (**B**) on adenylyl cyclase activity in nonactivated (open symbols, NA) and in mitogen-activated (filled symbols, A) cells. Values are mean ± SEM of duplicates obtained in four different experiments (n = 8). a, $P < 0.05$, b, $P < 0.01$.

Concerning SMS 201995 actions on adenylyl cyclase activity in Jurkat cells in the absence of mitogens, no significant ($F = 1$, $P = 0.46$) effect on the enzyme activity is seen in the whole range (1 pM to 10 nM) of the analog concentrations tested (Fig. 3A). However in the presence of mitogens, SMS 201995 significantly ($F = 8.18$, $P < 0.0015$) increases adenylyl cyclase activity. Such an increase of cAMP production is maximal at 10 nM concentration of SMS 201995 where it represents a three-fold increase over the adenylyl cyclase activity measured in the absence of SMS 201995 (Fig. 3A).

Conversely to SMS 201995, SRIF28 is not able to increase the adenylyl cyclase activity in either nonactivated or mitogen-activated cells. In the absence of mitogens, SRIF28 has no significant ($F = 0.19$, $P > 0.8$) effect on the enzyme activity (Fig. 3B). In mitogen-activated cells it significantly inhibits ($F = 7.06$, $P < 0.0027$) adenylyl cyclase activity in a dose-dependent manner. The maximal inhibition of about −50% is reached for the concentrations greater than 1 nM (Fig. 3B).

DISCUSSION

The most important finding of the present study is that octreotide SMS 201995 inhibits in vitro proliferation of Jurkat T cells. To our knowledge, this is the first report suggesting that this somatostatin analog might restrain the uncontrolled proliferation of leukemia T cells. In addition to the antiproliferative effects, SMS 201995 exerts stimulatory actions on cAMP production by adenylyl cyclase in activated Jurkat cells. Sensitivity of both cellular responses to SMS 201995 are compatible with the data we previously reported on the involvement of high-affinity sst3 receptors in somatostatin actions in Jurkat cells [Cardoso et al., 1998; Giannetti et al., 1999]. Besides, the overall increase of adenylyl cyclase activity by mitogens alone seen in the present study has already been reported previously, thus validating our experimental approach [Fratelli et al., 1989; Carlson et al., 1994].

The inhibitory actions of SMS 201995 on Jurkat-cell proliferation coupled to its capacity to increase the cAMP production shown here are in perfect agreement with the well recognized actions of this second messenger in the inhibition of normal and transformed T-cell proliferation [Tamir and Isakov, 1994]. Furthermore, we provide the experimental evidence for the opposite actions of SMS 201995 and SRIF28 on both parameters studied (i.e., proliferation and adenylyl cyclase activity). However, both SMS 201995 and SRIF28 modulate cell proliferation even at picomolar concentration, whereas somewhat greater concen-

trations of either analog are necessary to alter cAMP production. The reasons for these differences could be related to different experimental conditions used in the respective assays. Indeed, a 15-min assay period is sufficient to measure a rapid proximal response to agonist (e.g., receptor interaction with adenylyl cyclase). By contrast, a 5-h test period is required for quantification of a distal response such as cellular proliferation. The well recognized signal amplification along the transduction pathway from proximal to distal responses might therefore be in the origin of the apparent better efficiency of the agonists on cell proliferation than on adenylyl cyclase modulation. Nevertheless, the opposite coevolution of the observed actions of either agonist on cell proliferation versus adenylyl cyclase suggest that the causal relationship between proliferation and adenylyl cyclase activity (already reported by previous studies [Tamir and Isakov, 1994]) could account for the shown differential effects of SRIF analogs.

Comparison of adenylyl cyclase modulation by somatostatin analogs between the present analysis on whole Jurkat cells and our previous study on semipurified plasma membrane preparations points to some interesting differences. First, in semipurified membrane preparations from nonactivated cells, both SMS 201995 and SRIF28 inhibit adenylyl cyclase [Giannetti et al., 1999] whereas in whole cells both are inefficient. Second, in semipurified membrane preparations from activated cells, neither SMS 201995 nor SRIF28 have any significant effect on adenylyl cyclase activity [Giannetti et al., 1999], contrasting with their respective stimulatory and inhibitory actions observed here in whole cells. The reasons for these differences, seen even if the conditions of mitogen stimulation applied in both studies were rigorously identical, might be related to the use of different experimental paradigms. Indeed, the integrity of signaling systems in membrane preparations is obviously disturbed and the cross-talk reactions between them are altered or abolished. As a corollary, the cross-talk between transducing pathways operating in whole cells (but absent in membrane preparations) might either compensate or potentiate the direct inhibition of adenylyl cyclase by SMS 201995 and SRIF28 measured in the membrane preparations. Similarly, different agonist (angiotensin-II) actions have been previously reported on cAMP production in whole pituitary lactotroph cells on the one hand and in derived-membrane preparations on the other hand [Audinot et al., 1991]. Otherwise, because the inhibition of adenylyl cyclase activity via Giα depends on the nature and degree of the catalytic unit activation [Taussig et al., 1993], the presence of forskolin might be in the origin of the observed differences given that this pharmacologic tool was used in semipurified membrane experiments [Giannetti et al., 1999], but not in whole cell assays (present study).

An important fundamental question is raised by the present study. How two different agonists (SMS 201995 and SRIF28) with an almost identical affinity for sst3 receptor [Patel and Srikant, 1994] may elicit the opposite (stimulation and inhibition respectively) responses of the same effector (adenylyl cyclase) via the single (sst3) somatostatin receptor? The mechanisms of the agonist-specific triggering of effector responses remain unknown. However, the evidence in congruence with the hypothesis according to which different agonists may stabilize the formation of ternary (agonist/receptor/effector) complexes with different efficacies is growing [Gudermann et al., 1996]. For example, two variants of the rat pituitary adenylyl cyclase-activating polypeptide (PACAP-38 and PACAP-27) stimulate preferentially cAMP or inositol phosphate formation, respectively, through the same, PACAP-I receptor [Rawlings, 1994]. Alternatively, SMS 201995 and SRIF28 could differ in their capacity to induce receptor oligomerization, currently well documented for other receptors of G-protein coupled receptor family [Milligan, 1998]. Indeed, SMS 201995 but not SRIF28 binds to μ-opioid receptors [Maurer et al., 1982] and thus might induce sst3/μ-opioid receptor heterodimerization, resulting in a new complex receptor with properties different from those of sst3 receptor monomer. A similar κ/δ opioid receptor heterodimerization resulting in a new dimeric receptor with functional characteristics distinct from those of either receptors has been recently reported [Jordan and Devi, 1999].

In conclusion, taken together, our previous [Krantic et al., 1997; Cardoso et al., 1998; Giannetti et al., 1999] and present studies point to an important difference between native somatostatin analogs (SRIF14 and SRIF28) and the synthetic SMS 201995 analog on leukemia

T-cell proliferation. Thus, in contrast to SRIF14 and SRIF28 that are able to trigger a moderate increase in Jurkat-cell proliferation, SMS 201995 has the pronounced inhibitory actions on this cell response. SMS 201995 is already used routinely in the treatment of a number of pituitary and gastro-entero-pancreatic tumors [Lamberts et al., 1991; Reubi, 1997]. In particular, in cell lines derived from GH-secreting pituitary adenomas that are successfully treated clinically with SMS 201995, this analog when applied in vitro reduces both cell proliferation [Wah Cheung and Boyages, 1995] and GH secretion [Miller et al., 1995] to about 50%. By analogy, given that in our in vitro conditions, SMS 201995 inhibits leukemia cell line proliferation to the same extent, it is now worthwhile to compare the potential therapeutic benefit of SMS 201995 to those provided by classical chemotherapy of lympho-proliferative disorders.

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