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The two isoforms of the mouse somatostatin receptor (mSSTR2A and mSSTR2B) differ in coupling efficiency to adenylate cyclase and in agonist-induced receptor desensitization

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The somatostatin receptor 2 (mSSTR2) is alternatively spliced into two isoforms (mSSTR2A and mSSTR2B) which differ at the C-terminus. Both receptors bind somatostatin peptides with a similar high affinity when stably expressed in CHO-K1 cells. However, the spliced form (mSSTR2B) mediates a more efficient inhibition of adenylate cyclase and is much more resistant to agonist-induced reduction of binding than the longer form (mSSTR2A). These findings indicate that alternative splicing may be a physiological mechanism to modulate receptor desensitization and G-protein coupling of mSSTR2.

Somatostatin receptor 2 (mSSTR2); Isoform; Coupling; Adenylate cyclase; Desensitization

1. INTRODUCTION

Somatostatin (SS-14) and its N-terminally extended form SS-28 are widely distributed throughout the central nervous system and peripheral tissues. The peptides inhibit growth hormone release from the pituitary [1] and of insulin from the pancreas [2]. In the nervous system, they act as neurotransmitters or neuromodulators to inhibit neuronal firing [3], thus modulating complex behaviors, such as motor activity and cognition [4,5]. At the cellular level, the SS peptides act via receptors which are coupled to adenylate cyclase (AC) and ion channels by pertussis toxin sensitive G-proteins [6] resulting in inhibition of AC activity [7], reduction of calcium currents [8] and potentiation of potassium currents [4]. Two pharmacologically distinct SS receptors have been postulated earlier [9,10], but recently, five distinct receptor subtypes (SSTR1 to SSTR5) have been cloned [11–19]. In a previous study, we reported a further degree of diversification for the somatostatin receptor mSSTR2 which is alternatively spliced, generating two isoforms mSSTR2A and mSSTR2B. The two isoforms differ exclusively in the cytoplasmic C-terminus of the receptor, a region shown to be important for phosphorylation-dependent receptor desensitization [13].

The unspliced isoform (mSSTR2A) has been shown to bind the natural agonists SS-14 and SS-28 as well as the synthetic agonists SMS 201–995 (octeotride) and MK-678 (seglitide) with high affinity [11,13,20]. However, it is still unclear to which extent this isoform mediate the inhibition of AC. One group failed to observe any effect of mSSTR2A on AC after stable transfection into CHO-DG44 cells or transient transfection into COS-1 or HEK 293 cells [20,21] whereas others found an inhibition of AC after stable transfection into CHO-K1 cells [22].

In a preliminary study we observed that the mSSTR2B isoform mediates a strong inhibition of AC after stable transfection in CHO-K1 cells [23]. In the present study, we compare both the unspliced form mSSTR2A and the spliced isoform in their ability to mediate inhibiton of AC. In addition, since the C-terminus of G-protein coupled receptors has been shown to be important for phosphorylation-dependent receptor desensitization, we measured the loss of high affinity binding of both receptor isoforms after pretreatment with agonists. Finally, we constructed a C-terminally truncated mutant of mSSTR2A to further characterize the involvement of the C-terminus in the efficiency of AC inhibition and of receptor desensitization.

2. MATERIALS AND METHODS

2.1. *Cloning of expression vectors for mSSTR2A, mSSTR2B, 51YAST*

To generate a clone expressing mSSTR2B, a 1.7 kb *Bam*HI/*Eco*RV fragment of the clone 51CF3–1 [14] was subcloned into vector pcDNA I (Invitrogen, San Diego, USA). To clone the mSSTR2A receptor, PCR amplification of cDNA derived from DNAse-treated neuroblastomaxglioma cells (NG108–15) with sense primer SLP4 matching at nucleotides 554–575 and antisense-primer SLP5 matching at nucleotides 1707–1686 of non-translated regions of mSSTR2B (51CF3–1) was performed [14]. The PCR product of 1493 base pairs (51SCRI) was cleaved with *Xba*I and the resulting 1.2 kb fragment was subcloned into pcDNA I to obtain the clone 51YA-1E expressing exclusively the unspliced isoform mSSTR2A. For the construction of the C-terminally truncated receptor 51YAST, the PCR product 51SCRI

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was reamplified by PCR using the sense-primer SLP4 and the antisense-primer SLP6I (3'-TCATTCGTCCTGATTAGATCTG-5'). Primer SLP6I matches at nucleotides 34–55 of the intron of mSSTR2 [14] and introduces a stop-codon (ochre) at amino acid position 347 of mSSTR2A. A 1.1 kb *Xba*I fragment of 51YAST was subcloned into pcDNA I to obtain the expression clone 51YASTE. Sequencing of the expression clones was performed by the chain termination method [24].

2.2. *Stable expression of mSSTR2 receptors in CHO-K1 cells*

2 μg of the expression plasmids (51CF3–1E, 51YA-1E, 51YASTE) were contransfected with 0.1 μg of the plasmid pcDNA I neo (Invitrogen) into CHO-K1 cells (ATCC) using Lipofectin (BRL, Eggenstein, Germany). Stable transfectants were selected in NUT-F12 medium containing 600 μg/ml G418 (BRL) and 10% fetal calf serum (FCS). Clones expressing the highest levels of specific mRNA were selected and tested for binding of [^{125}I]Tyr^{11}-SS-14 (see below). Two to three clones of each receptor showing similar high affinity binding were used for further analysis.

2.3. *Binding assays*

3×10^5 stably transfected CHO-K1 cells were seeded in 35 mm six-well dishes in NUT-F12 medium containing 10% FCS. One day later, the cells were washed with 10 mM HEPES buffer (pH 7.4) and incubated with buffer containing 10 mM HEPES (pH 7.4), 5 mM $MgCl_2$, 0.5% (w/v) bovine serum albumin, 20 μg/ml bacitracin, 10,000 cpm [^{125}I]Tyr^{11}-SS-14 (Amersham, Braunschweig, Germany) and the indicated concentrations of SS-14 , SS-28 (Saxon Biochemicals, Hannover, Germany), SMS 201–995 (octreotide, Sandoz, Basle, Switzerland) or MK 678 (MSD, Munich, Germany) for 60 min at 8°C. Thereafter the cells were washed three times with ice-cold 10 mM HEPES buffer (pH 7.4) and dissolved in 1 ml of 8 M urea/3 M acetic acid. The radioactivity in the dissolved cells was measured in a γ-counter. Nonspecific binding was the binding of [^{125}I]Tyr^{11}-SS-14 in the presence of 1 μM SS-14. The IC_{50} values for the SS peptides to displace [^{125}I]Tyr^{11}-SS-14 from specific binding (= total binding minus nonspecific binding) were graphically determined by logit analysis. Agonist-induced loss of binding was tested as follows: stably transfected cells were washed twice with serum-free NUT-F12 medium (SFM) and pretreated with SFM containing 10 nM SMS 201–995 for 1 h at 27°C or 0°C. The cells were then washed three times with 10 mM HEPES buffer (pH 7.4) at room temperature followed by 60 min incubation with the binding buffer and [^{125}I]Tyr^{11}-SS-14 as described above.

2.4. *cAMP assay*

Accumulation of cAMP was measured as follows: 1.5×10^5 stably transfected CHO-K1 cells were seeded in 22 mm twelve-well dishes with NUT-F12 medium containing 10% FCS. The cells were then washed twice with SFM and pretreated with SFM containing 200 μM isobutylmethylxanthine for 30 min at 37°C. Thereafter, forskolin (25 μM final concentration) or a combination of forskolin and somatostatin peptides were added in the indicated concentrations and the incubation continued for 30 min at 37°C. the reaction was terminated by removing the medium, scraping and sonicating the cells in 1 ml of ice-cold HCl/EtOH (1 vol. of 1 N HCl/100 vols. EtOH). After evoparation of the solution, the residue was dissoved in TE buffer and the cAMP content measured by a radioassay kit (Amersham, Braunschweig, Germany).

3. RESULTS

The cloning and expression of the mSSTR2B receptor (346 amino acids) has been described [13].

For expressing the unspliced form of the mSSTR2 receptor (mSSTR2A; 369 amino acids) cDNA derived from NG108–15 cells mRNA was amplified with the primers SLP4 and SPL5 directed against noncoding regions of mSSTR2 (Fig. 1; and section 2). The resulting PCR product of 1493 bases (51SCRI) contains the coding region of mSSTR2A and the C-terminal sequences of mSSTR2B (Fig. 1). A 1.2 kb *Xba*I fragment of 51SCRI was subcloned into pcDNA I, generating the

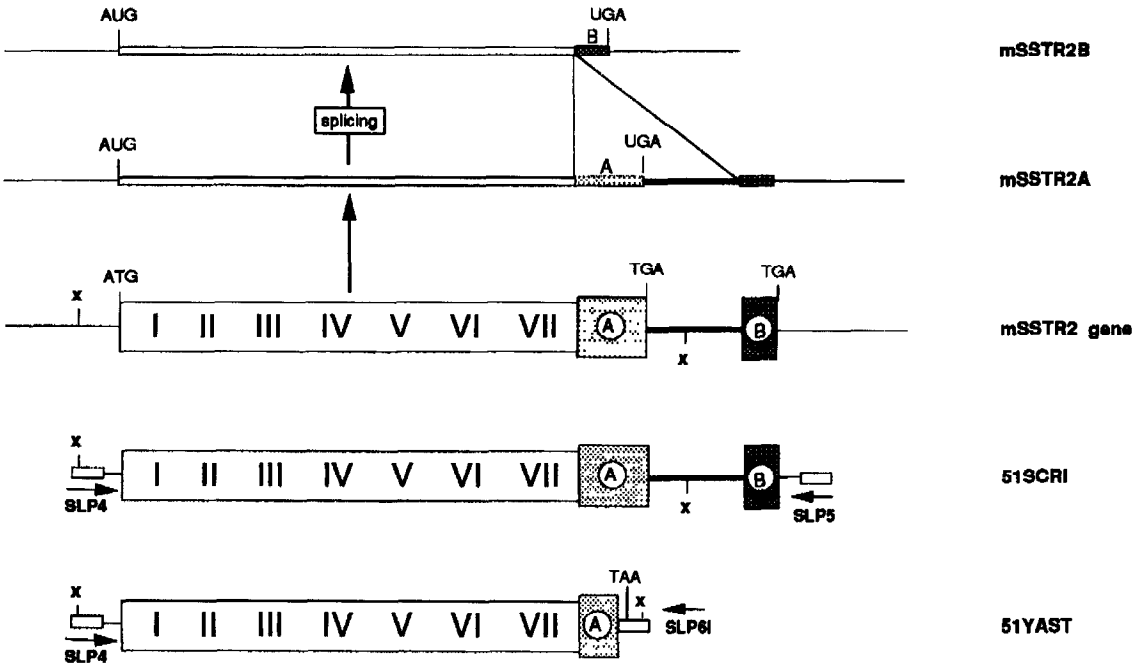

Fig. 1. Schematic representation of mSSTR2 gene, the mRNA coding for mSSTR2A and mSSTR2B, and the PCR amplification products 51SCRI and 51YAST. The C-terminus for mSSTR2A and mSSTR2B are depicted as shaded boxes. The primer SLP4 and SLP5 were used to PCR amplify 51SCRI. Primer SLP4 and SLP6I were used to PCR amplify the C-terminal truncated mutant receptor 51YAST. X indicates *Xba*I restriction sites.

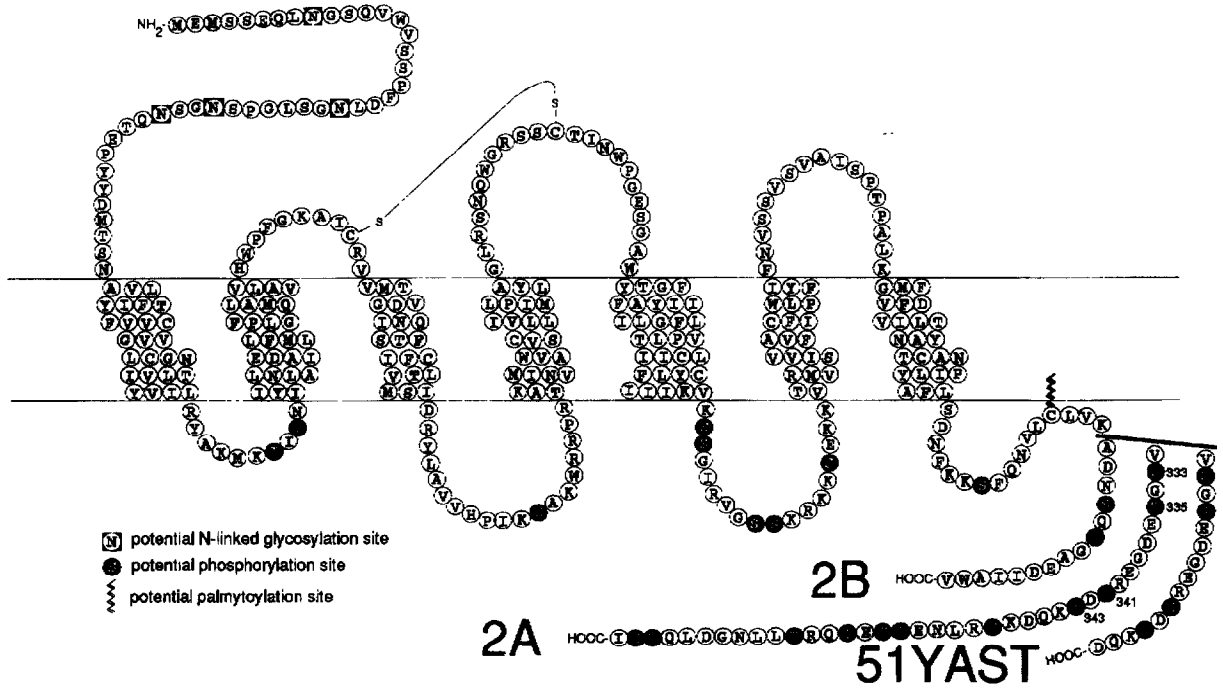

Fig. 2. Membrane topology of the spliced isoform mSSTR2B, mSSTR2A and the C-terminal truncated mutant 51YAST. mSSTR2B differs from mSSTR2A in the C-terminal amino acid sequence after the potential palmitoylation site. 51YAST is a mutant receptor derived from truncation of mSSTR2A to 346 amino acids.

expression clone 51YA-1E encoding exclusively the unspliced isoform mSSTR2A (369 amino acids).

The PCR product 51SCRI was reamplified using the primers SLP4 and SLP6I. Primer SLP6I introduced a *Xba*I restriction site and a stop mutation (ochre) at amino acid position 347 of mSSTR2A thus limiting the translation product to 346 amino acid (51YAST; Fig. 1). The 1.1 kb *Xba*I fragment was subcloned in pcDNA I generating the expression clone 51YASTE. The amino acid sequences of the three receptors are depicted in Fig. 2.

In a first set of experiments the functional properties of the spliced isoform mSSTR2B were studied after stable transfection into CHO-K1 cells. As seen in Fig. 3A, SS-14, SS-28, as well as the synthetic SS peptides SMS 201–995 and MK-678 displaced [^{125}I]Tyr^{11}-SS-14 from the newly expressed binding sites in CHO-K1 cells with a similar high affinity (IC_{50}s (nM): SS-14 0.58, SMS 201–995 0.71, MK-678 0.83, SS-28 0.92). The binding affinities are within the same range as those reported for the unspliced isoform mSSTR2A from mouse and rat [11,12,20].

The ability of mSSTR2B to inhibit AC activity is depicted in Fig. 3B. Forskolin (25 μM) treatment resulted in an about 5-fold increase of cAMP levels as compared to untreated cells. SS-14 and SS-28 mediated a dose-dependent inhibition of forskolin-stimulated cAMP accumulation by about 60% with an EC_{50} for SS-14 of 5.1 nM and for SS-28 of 4.2 nM). Interestingly, the synthetic peptides SMS 201–995 and MK-678 were more effective inhibitors of the AC than the natural SS-peptides. They completely inhibit forskolin-induced cAMP accumulation with EC_{50}s of about 0.9 nM for MK-678 and 1.1 nM for SMS 201–995 (Fig. 3B). In wild type CHO-K1 cells lacking somatostatin receptors, none of the agonists inhibited forskolin-stimulated cAMP levels (data not shown).

Co-incubation with 500 ng/ml pertussis toxin, a drug which uncouples receptors selectively from G_i-proteins and abolishes high affinity agonist binding to SSTR2 receptors [20], antagonized the inhibition of AC activity by SS-28 (Fig. 4), demonstrating that a pertussis toxin-sensitive G-protein couples the mSSTR2B receptor to AC.

In another series of experiments the pharmacological properties of mSSTR2B were compared with those of the unspliced isoform mSSTR2A and the C-terminally truncated receptor 51YAST after stable transfection into CHO-K1 cells (Fig. 5). In this experiment mSSTR2A, mSSTR2B and 51YAST bound SS-14 with

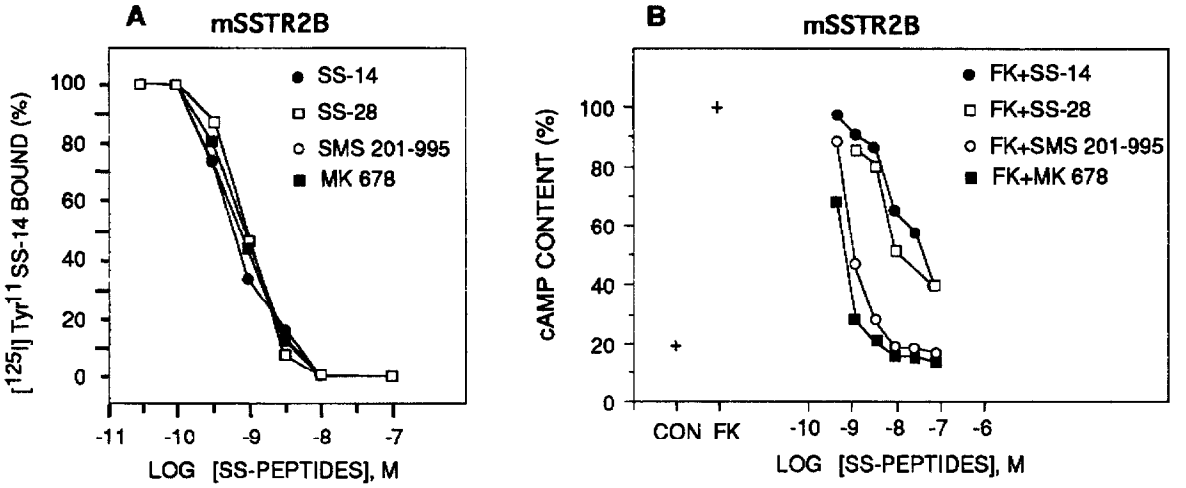

Fig. 3. Functional characterization of CHO-K1 cells expressing the spliced isoform mSSTR2B. (A) Binding of [^{125}I]Tyr^{11}-SS-14. The selected clone binds 1800 cpm in the absence and 300 cpm in the presence of 100 nM SS-14. (B) Dose dependence of inhibition of cAMP accumulation by different somatostatin agonists. cAMP levels were stimulated with 25 μM forskolin (FK) in the presence or absence of increasing amounts of SS peptides. All values are means of triplicate determinations and expressed in percent of maminum stimulation by forskolin.

similar high affinity as measured by displacement of [^{125}I]Tyr^{11}-SS-14 with SS-14 (IC_{50}s (nM): 0.51, 0.62, 0.72, respectively), demonstrating that the C-terminus of the receptors has no major effect on high affinity agonist binding.

As seen in Fig. 5B all three receptors mediated the inhibition of forskolin-stimulated AC by SS-14; however, to a different degree. A dose of 100 nM SS-14 inhibited forskolin-stimulated AC in cells expressing mSSTR2B by 61% and 51YAST by 55%, but was less effective in blocking forskolin-stimulated AC in cells expressing mSSTR2A (only about 33%). A similar observation was obtained in two additional experiments with other independently transfected CHO-K1 cells. Inhibition of forskolin-stimulated AC by 100 nM SS-14 (mean ± S.E.M. of 3 experiments): mSSTR2B 57 ± 9; mSSTR2A 31 ± 10; 51YAST 53 ± 12; $P < 0.05$ for mSSTR2B vs. mSSTR2A and mSSTR2B vs. 51YAST; Student's t-test). These findings indicate that the length of the C-terminus can affect the coupling to AC.

Somatostatin receptors have been shown to desensitize when chronically exposed to agonists [25]. Exposure of CHO-K1 cells expressing mSSTR2A and 51YAST to 10 nM of SMS 201–995 for 1 h at 27°C caused a marked decrease of high affinity binding of [^{125}I]Tyr^{11}-SS-14 of 70–80% compared to nonpretreated cells (Fig. 5C). Pretreatment of the cells with SMS 201–995 at 0°C did not result in a diminished binding (data not shown) indicating that the SS peptide used for pretreatment had been removed effectively under the washing conditions. In contrast, a similar pretreatment of cells expressing the spliced isoform mSSTR2B caused only a 35% loss of [^{125}I]Tyr^{11}-SS-14 binding.

The time-course of agonist-dependent loss of [^{125}I]Tyr^{11}-SS-14 high affinity binding is illustrated in Fig. 6. CHO-K1 cells expressing mSSTR2A or mSSTR2B were pretreated with 10 nM SMS 201–995 for 0 min, 5 min, 20 min or 60 min followed by removal of the agonist and subsequent determination of high affinity binding of [^{125}I]Tyr^{11}-SS-14. As compared to control (0 min), a 45% loss of high affinity [^{125}I]Tyr^{11}-SS-14 binding to mSSTR2A occurred within 5 min of receptor occupation by SMS 201–995. Within 20 min, loss of high affinity binding of [^{125}I]Tyr^{11}-SS-14 to mSSTR2A was almost maximal (about 80%). The re-

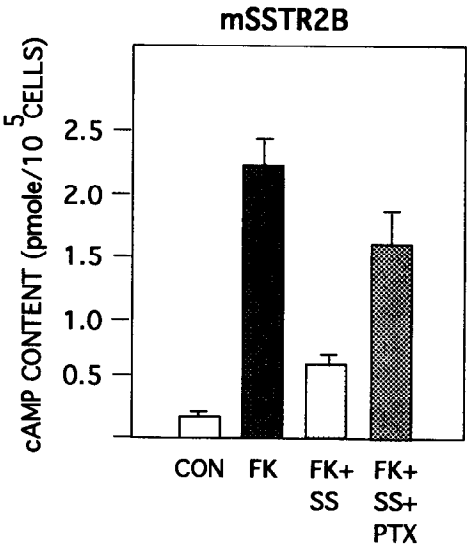

Fig. 4. Effect of pertussis toxin on inhibition of forskolin-stimulated cAMP accumulation in CHO-K1 cells expressing the spliced isoform mSSTR2B. Forskolin (FK; 25 μM)-stimulated cAMP accumulation was inhibited by 1 μM SS-28 (FK + SS). Co-incubation with pertussis toxin (PTX; 500 ng/ml) diminished this inhibition (FK + SS + PTX) by uncoupling the pertussis toxin-sensitive G-protein from the receptor. CON = untreated cells.

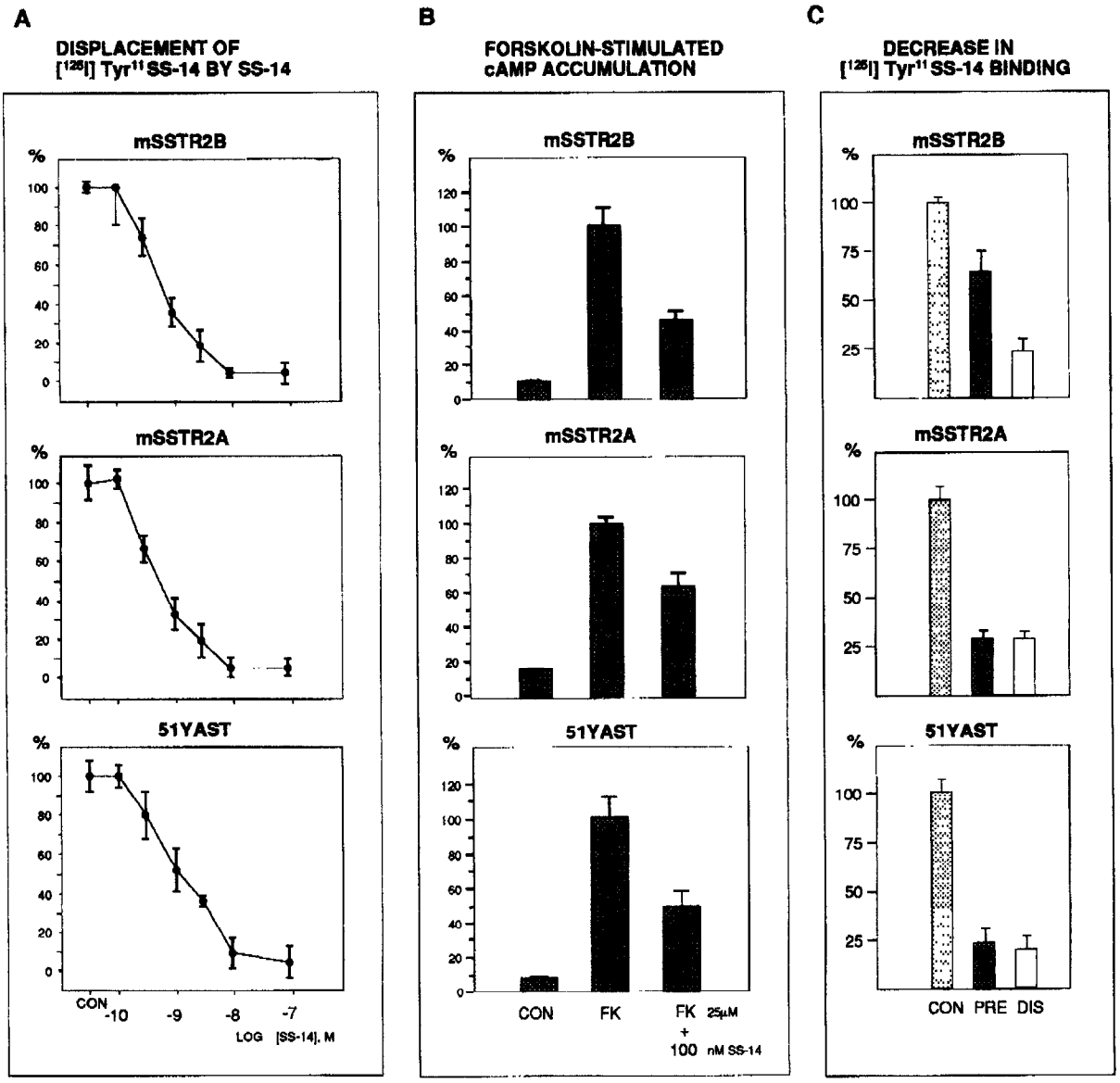

Fig. 5. Comparison of the isoform mSSTR2B, mSSTR2A and the mutant receptor 51YAST stable expressed in CHO-K1 cells with regard to agonist binding, inhibition of adenylyl cyclase activity and agonist-dependent loss of binding. (A) Displacement of high-affinity binding of [^{125}I]Tyr^{11}-SS-14 to the receptors by increasing amounts of SS-14. (B) Inhibiton of forskolin-stimulated cAMP accumulation (FK) by 100 nM SS-14. (C) Agonist-dependent receptor desensitization: pretreatment of CHO-K1 cells stably expressing mSSTR2B, mSSTR2A or 51YAST with 10 nM SMS 201–995 for 1 h at 27°C (PRE) caused a decrease of high-affinity [^{125}I]Tyr^{11}-SS-14 binding to the receptor, as compared to nonpretreated cells (CON). DIS shows the displacement of [^{125}I]Tyr^{11}-SS-14 binding to nonpretreated cells with 10 nM of SMS 201–995. All values are means of triplicate determinations and expressed in percent of control (CON) binding.

duction of [^{125}I]Tyr^{11}-SS-14 binding to mSSTR2B exhibited a similar time-course, but was much less pronounced. Only 30–35% of high affinity binding of [^{125}I]Tyr^{11}-SS-14 were lost after 20–60 min pretreatment with SMS 201–995.

4. DISCUSSION

In the present study the pharmacological properties of the two isoforms of the somatostatin receptor subtype 2 (mSSTR2A and mSSTR2B) after expression in CHO-K1 cells have been compared. Evidence was provided that both isoforms exhibit a similar binding affinity to SS-14, but differ in their ability to inhibit AC and to loose binding upon treatment with agonist.

The unspliced form mSSTR2A has previously been characterized after stable transfection into CHO-DG44 or transient transfection into COS-1 and HEK 293 cells [11,20,21]. In these cells, however, mSSTR2A bound SS peptides with a high affinity, but failed to inhibit forskolin-stimulated cAMP accumulation [20]. In contrast, when stably transfected into CHO-K1 cells we consis-

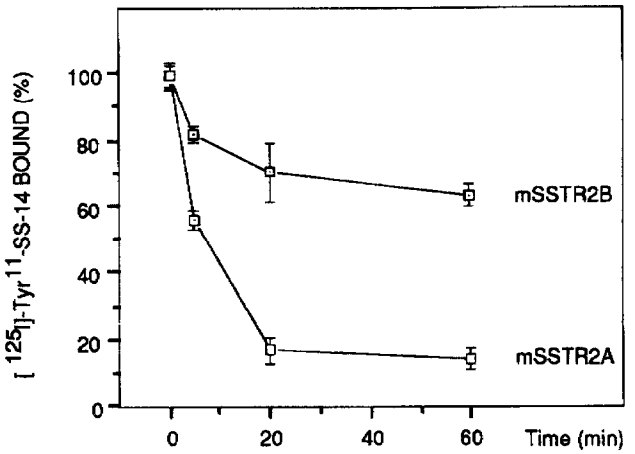

Fig. 6. Time course of agonist-dependent loss of binding to the unspliced isorform mSSTR2A and the spliced isoform mSSTR2B. High affinity [^{125}I]Tyr^{11}-SS-14 binding to CHO-K1 cells expressing mSSTR2A and mSSTR2B was measured following pretreatment for 0, 5, 20 or 60 min with 10 nM SMS 201–995.

tently obtained an inhibition of forskolin-stimulated AC by SS peptides (Fig. 5). Similar results were recently obtained by Hadcock and coworkers after expression of rat mSSTR2A in CHO-K1 cells [22].

A possible explanation for these discrepant results is that different G-proteins are expressed in the cells used for transfection. Thus, the CHO-DG44 cell line expresses $G_{i\alpha3}$, but not $G_{i\alpha1}$, $G_{i\alpha2}$ or $G_{o\alpha}$ [20]. In contrast, $G_{i\alpha1}$, $G_{i\alpha2}$, but not $G_{i\alpha3}$ and $G_{o\alpha}$ were detected in CHO- K1 cells [26]. In addition, since $G_{i\alpha1}$ has been shown to be necessary for coupling SRIF receptors to AC in AtT-20 cells [27], it is possible that the lack of $G_{i\alpha1}$ in CHO-DG44 is one of the reasons for the failure of transfected mSSTR2A to mediate inhibition of AC in these cells. However, the absence of $G_{i\alpha1}$ cannot be solely responsible for the inability of mSSTR2A to inhibit AC activity in CHO-DG44 cells, since COS-1 cells which express all subtypes of $G_{i\alpha}$ subunits and HEK 293 cells which express $G_{i\alpha1}$ also failed to mediate SS inhibition of AC [20,21].

Both isoform mSSTR2A and mSSTR2B are negatively coupled to AC in transfected CHO-K1 cells; however, the isoform mSSTR2B was more efficient, displaying a higher inhibition of forskolin-stimulated cAMP accumulation. Moreover, C-terminal truncation of mSSTR2A to the same length as mSSTR2B (mutant receptor 51YAST) improved the ability of the receptor to inhibit AC activity compared to mSSTR2A (Fig. 5B), indicating that the efficiency of negative coupling to AC is dependent from the length of the C-terminus of mSSTR2. Although it has been shown that the third intracellular loop contains the primary regions responsible for specificity in receptor G-protein interactions [28], the C-terminus may act in concert to obtain full efficiency in receptor G-protein coupling. It is noteworthy in this context that a variety of receptors which inhibit AC (D_2, dopaminergic; α_2, adrenergic; A_1, purinergic; M_2, muscarinergic) possess a short C-terminus [29].

An interesting finding was that in CHO-K1 cells expressing mSSTR2B the effectiveness of the stable SS peptides SMS 201–995 and MK-678 in inhibiting AC was higher than that of the natural occurring peptides SS-14 and SS-28. Since the binding properties of the peptides were similar, it appears that the peptide differ in their ability to cause binding-induced conformational changes of mSSTR2B which, in turn, affects coupling to AC.

Previous studies have shown that mSSTR2A transfected in CHO-DG44 cells exhibit a marked loss of agonist binding after pretreatment with SS-14 [20]. A similar observation was made in CHO-K1 cells expressing mSSTR2A (Fig. 5C; Fig. 6) which showed a highly diminished agonist binding by about 80% after pretreatment with 10 nM SMS 201–995 for 60 min. The loss of binding was much less pronounced (about 30%) in CHO-K1 cells transfected with the spliced isoform mSSTR2B. On the other hand, cells expressing the C-terminally truncated receptor 51YAST showed a similar loss of agonist binding after pretreatment with SMS 201–995 as those expressing mSSTR2A. This indicates that the molecular requirements for the agonist-induced decrease in binding reside within the C-terminal 15 amino acids of 51YAST which are present in mSSTR2A, but not in mSSTR2B.

Previous studies have shown that a prolonged treatment with SS analogs of AtT-20 pituitary cells which express mSSTR2A and mSSTR2B [13,30] desensitize the cells to inhibition of hormone release and cAMP formation [25]. This desensitization was associated with a reduced SS binding which most probably resulted from an uncoupling of the receptor from G-proteins [25]. Uncoupling of the β_2 receptor from G_s-protein has been shown to involve phosphorylation by βARK (β_2 adrenergic receptor kinase) [31] at serine and threonine residues in the C-terminus of the receptor. Interestingly, evidence has been provided that βARK is involved in the phosphorylation of SS receptors in S49 lymphoma cells which occurs upon desensitization to SS-14 [32]. We suggest, that phosphorylation of Ser^{333}, Thr^{335}, Ser^{341} and Ser^{343} (Fig. 2) which are common for 51YAST and mSSTR2A but not mSSTR2B might be responsible for the profound desensitization of these receptors, since 51YAST behaves similar to mSSTR2A (Fig. 5C) in agonist-dependent loss of binding.

The presence of both isoforms in brain and pituitary tissues of various species ([13,30]; Vanetti et al., in preparation) indicates that the alternative splicing may be a physiologiscal mechanism to modulate the coupling efficiency or desenzitation of mSSTR2.

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