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Urotensin II and urotensin II-related peptide activate somatostatin receptor subtypes 2 and 5

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ABSTRACT

The UII and urotensin II-related peptide (URP) genes belong to the same superfamily as the somatostatin gene. It has been previously shown that somatostatin activates the UII-receptor (UTR). In contrast, the possible interaction between UII and URP and somatostatin receptors has remained scarcely analyzed. Herein, we have investigated the effects of UII and URP on cell proliferation and free cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) in CHO-K1 cells stably expressing the porcine somatostatin receptor subtypes sst2 and sst5. Results show that both UII and URP induce stimulation of cell proliferation mediated by sst2 receptors and UII provokes inhibition of cell proliferation mediated by sst5 receptors. UII and URP also provoked an increase of $[Ca^{2+}]_i$ in both sst2- and sst5-transfected cells. Together, our present data demonstrate that UII and URP directly activate sst2 and sst5 and thus mimic the effect of somatostatin on its cognate receptors.

1. Introduction

Urotensin II (UII) is a cyclic neuropeptide first isolated from the teleost urophysis [41] and subsequently identified in several vertebrate species, from frog to human (reviewed in Ref. [48,49]). UII acts through binding to the G protein-coupled receptor GPR14 [4,27,36,37], with seven transmembrane domains, now referred to as the UII receptor (UTR) [15]. Both UII and UTR are expressed in a variety of tissues and cell types, including the brain and spinal cord as well as in kidney, liver, pancreas, adrenal gland, and vascular endothelial cells [1,22,33,46,47]. Indeed, UII has been demonstrated to play multiple physiological functions, from the regulation of the cardiovascular and renal systems to the control of insulin secretion and sleep [5,10,20,39].

In 2003, Sugo et al. [47] isolated a novel peptide from the rat brain sharing seven of its eight amino acids with UII, which was demonstrated to be encoded by a separate gene, and was named urotensin II-related peptide (URP). Furthermore, URP was found to bind and activate the UTR, thus leading to the proposal that URP is an endogenous and functional ligand for the UTR [47]. Likewise, URP is expressed in the central nervous

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system as well as in peripheral tissues such as ovary, testis and placenta [35,47].

The cyclic hexapeptide core of UII and URP shares substantial sequence similarities with the biologically active region of somatostatin and its paralog cortistatin [6,14,41,47], so that UII was originally described as a somatostatin-like peptide [41]. The chromosomal localization of UII, URP, somatostatin and cortistatin has been recently determined in several vertebrate species by radiation hybrid mapping and *in silico* sequence analysis [48] and it has been found that the genes encoding URP and somatostatin on the one hand, and UII and cortistatin on the other hand are physically linked, indicating that all four genes derive from a common ancestral gene. In addition, UTR exhibits high sequence identity with somatostatin receptors [31]. Actually, UTR is located in the same chromosomal region as sst3, at 17q23, suggesting that these two receptors arose by tandem duplication [48]. In line with these observations, both somatostatin and cortistatin can activate UTR, yet with lower efficacy than UII [28], and somatostatin receptor antagonists have been employed to design UII antagonists [11]. In contrast, there is currently limited information available as to whether UII and URP can act at the somatostatin receptors. To address this question, we have herein investigated the effects of UII and URP on cell proliferation and free cytosolic Ca^{2+} concentrations ($[Ca^{2+}]_i$) in Chinese hamster ovary (CHO)-K1 cells stably expressing the somatostatin receptor subtypes sst2 and sst5.

2. Materials and methods

2.1. Peptides and test substances

SRIF-14 was purchased from Neosystem (Strasbourg, France). Agonists for somatostatin receptor subtypes (sst1: L-779,591; sst2: L-779,976; sst3: L-796,778; sst4: L-803,087; sst5: L-817,818) were kindly provided by Dr. Susan P. Rohrer (Merck Research Co., NJ). Human UII (hUII, ETPDCFWKYCV) and URP (ACFWKYCV) as well as the UII analog [$Ser^{5,10}$]$hUII_{4-11}$ (Ser-UII) were synthesized by the solid phase methodology on a Pioneer PerSeptive Biosystem peptide synthesizer (Applera France, Courtaboeuf, France) using the Fmoc procedure as previously described [13,23]. All peptides were purified on a 2.2× 25 cm Vydac C18 column (Alltech, Templemars, France) (>98% pure) and characterized by MALDI-TOF MS on a Voyager DE-PRO mass spectrometer (Applera France). Palosuran (ACT-058362; 1-[2(4-benzyl-4-hydroxy-piperidin-1-yl)-ethyl]-3-(2-methyl-quinolinb-4-yl)-urea sulfate salt) was kindly provided by Dr. M. Clozel (Actelion Pharmaceuticals Ltd., Switzerland).

2.2. Generation of porcine ssts (pssts) recombinant expression vectors

Genomic DNA was extracted from swine Large-White/Landrace pituitaries, using the Tripure Isolation Reagent (Boehringer Mannheim, Mannheim, Germany) according to the manufacturer's instructions. The coding sequence of psst5A was amplified by PCR from genomic DNA using the following specific primers designed at the ATG and TGA region (GenBank accession no. AY156052): S_sst5_ATG: 5′-GCCGTGATGGAGC-CTCTGTTCC-3′ and AS_sst5_TGA: 5′-CCTCACAGCCTGCTGG-TCT-3′. PCR fragments were reamplified with primers including the restriction sites HindIII and EcoRI (S_psst5_ATG_HindIII: 5′-AAGCTTGCCACAGCCGTGATGGAGCCTCT-3′ and AS_psst5_TGA_EcoRI: 5′-GAATTCCCTCACAGCCTGCTGG-TCT-3′). Then, the resulting sequence was inserted into the corresponding restriction sites of the expression vector pCDNA3.1+ (Invitrogen, Barcelona, Spain). The porcine sst2 cDNA (GenBank accession no. AY138806) was cloned as described elsewhere [16]. Identity of the cloned sequences was confirmed by sequencing (Central Sequencing Service, University of Cordoba, Spain).

2.3. Stable expression of psst2 and psst5 in CHO-K1 cells

CHO-K1 cells were cultured to semiconfluence in 12-well plates using F12 medium supplemented with 10% FBS and 1% antibiotic-antimycotic solution, and transfected with 1.5 μg of the recombinant plasmids, using Lipofectamine 2000™ (Gibco, Barcelona, Spain), as described previously for psst2 [16]. Briefly, 24 h after transfection, medium was replaced by fresh F12 supplemented with 1 mg/ml geneticin (G418; Gibco). One week later, surviving cells were detached, diluted to 70 cells/ml and plated on 96-well plates at 0.7 cells/well. Monoclonal cell lines expressing each recombinant psst were followed daily by contrast phase microscopy. All cultures were routinely maintained in the presence of the selection agent G418 sulfate (1 mg/ml) at 37 °C in humidified air containing 5% CO_2. Cells were detached and re-seeded when they reached 95% confluence. Levels of receptor density remained constant over the duration of the study (data not shown).

2.4. Determination of cell proliferation

Cell proliferation was evaluated in parallel in psst stably transfected cells and in CHO-K1 cells stably transfected with the pCDNA3.1 empty vector by measuring incorporation of [^{3}H]thymidine to genomic DNA. To this end, cells were seeded at 150,000 cells/well in 12-well plates and cultured for 72 h at 37 °C in F12 containing 10% FBS and 1 mg/ml geneticin. Cells were washed and incubated for 24 h in medium without FBS for growth arrest. Thereafter, cells were washed again with fresh medium and incubated for an additional 24 h period in F12 containing 0.25 μCi [^{3}H]thymidine (Amersham Pharmacia Biotech, Aylesbury, UK) and the corresponding test substances. Specifically, cultures were exposed to somatostatin or the specific non-peptidic sst agonists at doses ranging from 10^{-15} to 10^{-7} M, UII and URP at doses ranging from 10^{-10} to 10^{-6} M, or 10^{-6} M of the inactive UII analog Ser-UII. In addition, the effect of 10^{-8} M of the UTR antagonist palosuran, alone or in combination with UII or URP (10^{-6} M), was tested. After treatments, cells were washed with ice cold NaCl (0.9%) and trichloroacetic acid (5%) and then lysed for 2 h at room temperature in 1 ml of 1N NaOH. Acid-insoluble [^{3}H]thymidine was quantified in a liquid scintillation counter (LS 6000TA, Beckman Coulter, Fullerton, CA). All the experiments were performed in triplicate, and each experiment was repeated at least 3 times. Results are expressed as percentages of basal values in non-treated cultures (100%).

2.5. Measurement of free cytosolic calcium concentration ($[Ca^{2+}]_i$) in single cells

Stably transfected CHO-K1 cells plated on glass coverslips 24 h before the assay were incubated for 30 min at 37 °C with 2.5 μM Fura-2 AM (Molecular Probes, Eugene, OR) in phenol red-free DMEM containing 20 mM $NaHCO_3$, pH 7.4. Coverslips were washed with phenol red-free DMEM and mounted on the stage of a Nikon Eclipse TE2000 E microscope (Nikon, Tokyo, Japan) fitted with a back thinned-CCD cooled digital camera (ORCA II BT; Hamamatsu Photonics, Hamamatsu, Japan). Cells were examined under a 40× oil immersion objective during exposure to alternating 340 and 380 nm light beams, and the intensity of light emission at 505 nm was measured every 5 s. Changes in $[Ca^{2+}]_i$ after administration of UII (10^{-6} to 10^{-7} M) or URP (10^{-6} to 10^{-7} M) were recorded as background subtracted ratios of the corresponding excitation wavelengths (F340/F380) using the MetaFluor software (Imaging Corporation, West Chester, PA). Where indicated, cells were preincubated for 30 min with 10^{-8} M palosuran.

2.6. Statistical analysis

Data are expressed as mean ± S.E.M. obtained from the number of independent experiments indicated in each figure. For $[Ca^{2+}]_i$ measurements, at least 100 cells were analyzed per experimental condition. Statistical analysis was carried out using one-way ANOVA followed by a Newman–Keul's statistical test for multiple comparisons. Differences were considered to be significant at $P < 0.05$.

3. Results

3.1. Control of cell proliferation in CHO cells expressing psst2 by SRIF, SRIF agonists, UII and URP

We first analyzed the dose-related effect of SRIF treatment on cell proliferation through specific activation of psst2 (Fig. 1A). At the highest concentration tested (10^{-7} M), SRIF elicited a significant stimulatory effect on [^{3}H]thymidine incorporation (176.2 ± 12.1%, $P < 0.05$). At a lower concentration (10^{-9} M), SRIF also induced a slight increase in cell proliferation, yet this increase did not reach statistical significance (153.6 ± 31.3%; $P > 0.05$). Likewise, SRIF doses below 10^{-9} M did not induce significant variations in cell proliferation.

In line with the results found for SRIF, a non-peptidic sst2 specific agonist, L-779,976, also caused a stimulatory effect on [^{3}H]thymidine incorporation in psst2-expressing CHO-K1 cells, which was of higher magnitude than that elicited by

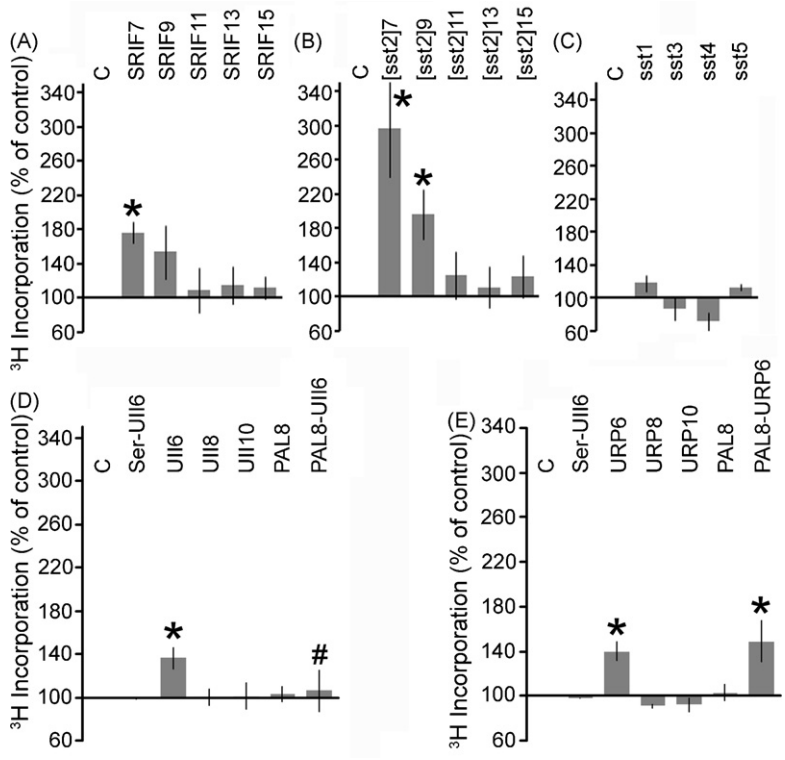

Fig. 1 – Effect of somatostatin (SRIF), sst receptor agonists, UII and URP on psst2-receptor-driven cell proliferation. (A and B) Concentration-dependent effect of SRIF (A) and the sst2 receptor agonist L-779,976 (B) on [^{3}H]thymidine incorporation in CHO-K1 cells stably expressing the psst2 receptor. (C) Effect of the sst1 (L-779,591), sst3 (L-796,778), sst4 (L-803,087) and sst5 (L-817,818) receptor agonists on [^{3}H]thymidine incorporation in psst2-expressing CHO-K1 cells. SRIF (10^{-7} M) and the sst2-receptor agonist (10^{-9} and 10^{-7} M) elicited significant stimulatory effect on cell proliferation through psst2-receptor activation, whereas other sst-receptor agonists are devoid of effect. (D and E) Concentration-dependent effect of UII (D) and URP (E) on [^{3}H]thymidine incorporation in psst2-expressing CHO-K1 cells. In both cases, the highest concentration of peptides tested (10^{-6} M) significantly stimulated CHO-K1 proliferation. The UTR antagonist palosuran, suppressed the proliferative effect of UII but did not inhibit that of URP. *, $P < 0.05$ *vs.* C. #, $P < 0.05$ *vs.* 10^{-6} M UII.

SRIF (Fig. 1B). Thus, 10^{-7} M and 10^{-9} M L-779,976 significantly increased cell proliferation (297.1 ± 56.7% and 196.0 ± 28.9%, respectively; $P < 0.05$). In clear contrast, administration of a high concentration (10^{-7} M) of the specific agonists for the other ssts failed to elicit any stimulatory effect on cell proliferation (Fig. 1C).

Next, we analyzed the effect of increasing doses of UII and URP on cell proliferation in psst2-expressing CHO cells. As shown in Fig. 1D, 10^{-6} M UII induced a significant increase of [^{3}H]thymidine incorporation (136.0 ± 10.2%; $P < 0.05$). On the other hand, [^{3}H]thymidine incorporation was unaffected by treatment with lower doses of UII. Furthermore, pre-treatment of psst2-expressing CHO-K1 cells with the UTR antagonist palosuran, which had no effect by itself on basal [^{3}H]thymidine incorporation, resulted in complete abrogation of 10^{-6} M UII-induced increase of cell proliferation (Fig. 1D).

Similar to UII, 10^{-6} M URP (but not lower doses) also caused a significant increase in [^{3}H]thymidine incorporation in psst2-expressing cells (140.2 ± 8.6%; $P < 0.05$; Fig. 1E). However, contrarily to that found for UII, this effect was not altered by pre-treating psst2-expressing CHO-K1 cells with 10^{-8} M palosuran (Fig. 1E).

As an additional control, we employed the inactive form of UII, Ser-UII, which has been previously reported to lack activity on rat aortic ring contraction [21]. Accordingly, administration of this synthetic peptide for 24 h did not induce significant changes in cell proliferation on psst2-expressing CHO-K1 cells (Fig. 1D and E).

Additionally, cells stably transfected with the empty vector (mock transfection) were developed and subjected to the same treatments and at the same concentrations with the different substances tested as CHO-K1 cells stably expressing pssts, to establish whether the effect of these substances on cell proliferation is directly mediated by their interaction with the receptors. No effect was found on mock-transfected cells in any case and under any condition analyzed herein (data not shown), thereby indicating that the effects observed on CHO cell proliferation are specifically mediated by binding of SRIF, UII, or URP to pssts.

3.2. *Control of cell proliferation in CHO cells expressing psst5 by SRIF, SRIF agonist, UII and URP*

We also analyzed the variations on the proliferation rate mediated by activation of psst5 and found that 10^{-7} M and

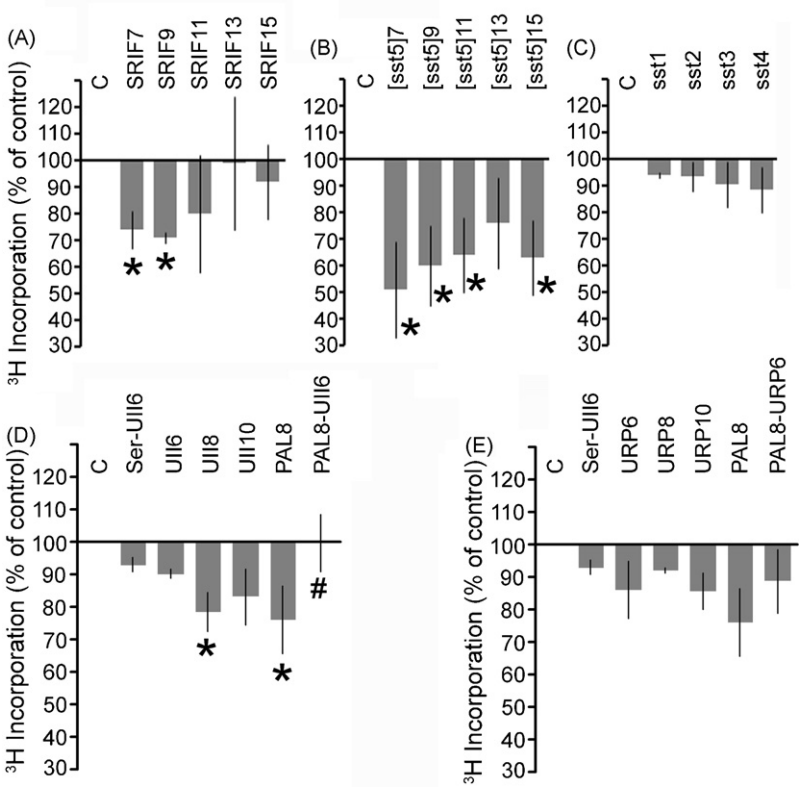

Fig. 2 – Effect of somatostatin (SRIF), sst receptor agonists, UII and URP on psst5-receptor-driven cell proliferation. (A and B) Concentration-dependent effect of SRIF (A) and the sst5 receptor agonist L-817,818 (B) on [^{3}H]thymidine incorporation in CHO-K1 cells stably expressing the psst5 receptor. (C) Effect of the sst1 (L-779,591), sst2 (L-779,976), sst3 (L-796,778) and sst4 (L-803,087) receptor agonists on [^{3}H]thymidine incorporation in psst5-expressing CHO-K1 cells. SRIF (10^{-9} and 10^{-7} M) significantly inhibited [^{3}H]thymidine incorporation in CHO-K1 cells stably expressing psst5 receptor. Likewise, at all concentrations tested, but 10^{-13} M, the sst5 specific agonist inhibited [^{3}H]thymidine incorporation. In contrast, cells were unresponsive to agonists specific for the other ssts. (D and E) Concentration-dependent effect of UII (D) and URP (E) on [^{3}H]thymidine incorporation in psst5-expressing CHO-K1 cells. At a concentration of 10^{-8} M, UII elicited an inhibitory effect on cell proliferation. Intriguingly, when administered alone, the UII-receptor antagonist palosuran exhibited an inhibitory effect on [^{3}H]thymidine incorporation in stably transfected psst5 CHO-K1 cells. *, $P < 0.05$ *vs.* C. #, $P < 0.05$ *vs.* PAL8.

10^{-9} M SRIF were able to elicit a significant inhibitory effect on cell proliferation in the clonal psst5-CHO-K1 cell line (74.4 ± 7.2% and 70.9 ± 2.0%, respectively; $P < 0.05$; Fig. 2A). Furthermore, at all doses tested, the sst5 specific agonist L-817,818, but 10^{-13} M, significantly inhibited [^{3}H]thymidine incorporation, the effect being maximal at a concentration of 10^{-7} M (51.1 ± 17.6%; $P < 0.05$; Fig. 2B). In contrast, cells were not responsive to agonists specific for the other ssts (Fig. 2C).

Regarding the effect of UII and URP on cell proliferation through their action on the psst5 receptor subtype (Fig. 2D and E), only 10^{-8} M UII was capable to alter the proliferation rate of CHO cells stably expressing psst5 (78.3 ± 6.1%; $P < 0.05$; Fig. 2D). This inhibitory effect was completely abolished by incubation of cells with 10^{-8} M palosuran (Fig. 2D). Intriguingly, when administered alone the UTR antagonist exhibited a significant inhibitory effect on [^{3}H]thymidine incorporation in stably transfected psst5 cells (75.9 ± 10.4%; $P < 0.05$; Fig. 2).

3.3. *Measurement of $[Ca^{2+}]_i$ in stably transfected psst2 CHO-K1 cells in response to UII and URP*

We also evaluated the effect of UII and URP on $[Ca^{2+}]_i$ dynamics in single cells stably transfected with the psst2 construct. As shown by the representative example illustrated in Fig. 3A, administration of 10^{-6} M UII provoked a rapid increase in $[Ca^{2+}]_i$ in CHO-K1 cells expressing psst2. Specifically, 74.2% of cells (n = 89 responsive cells out of 120 total cells) responded to 10^{-6} M UII by a significant $[Ca^{2+}]_i$ increase above baseline (195.6 ± 19.0%; $P < 0.05$). A lower dose of the peptide (10^{-7} M) also significantly increased $[Ca^{2+}]_i$ in these cells (177.5 ± 8.7%; $P < 0.05$), although the proportion of responsive cells (25%; n = 27 out of 108 cells) was lower than that observed for 10^{-6} M UII.

Analysis of the effect of palosuran in psst2-expressing cells revealed that, when administered alone, this antagonist evoked a stimulatory response on $[Ca^{2+}]_i$ (214.9 ± 34.2%; $P < 0.05$) in 20.2% of cells (n = 20 out of 101 cells). In contrast, and as occurred with the proliferation rate, pre-incubation of cells with palosuran prevented any increase in $[Ca^{2+}]_i$ in response to 10^{-6} M UII in all cells tested (Fig. 3B).

As for UII, 10^{-6} M URP also increased $[Ca^{2+}]_i$ in stably transfected psst2 cells (Fig. 3C), though its effect was slightly stronger than that evoked by UII, both in terms of the percentage of responsive cells (84.6%; n = 142 responsive cells out of 168 cells) and in the maximal $[Ca^{2+}]_i$ reached (304.5 ± 28.9%; $P < 0.05$). Likewise, as that observed when a

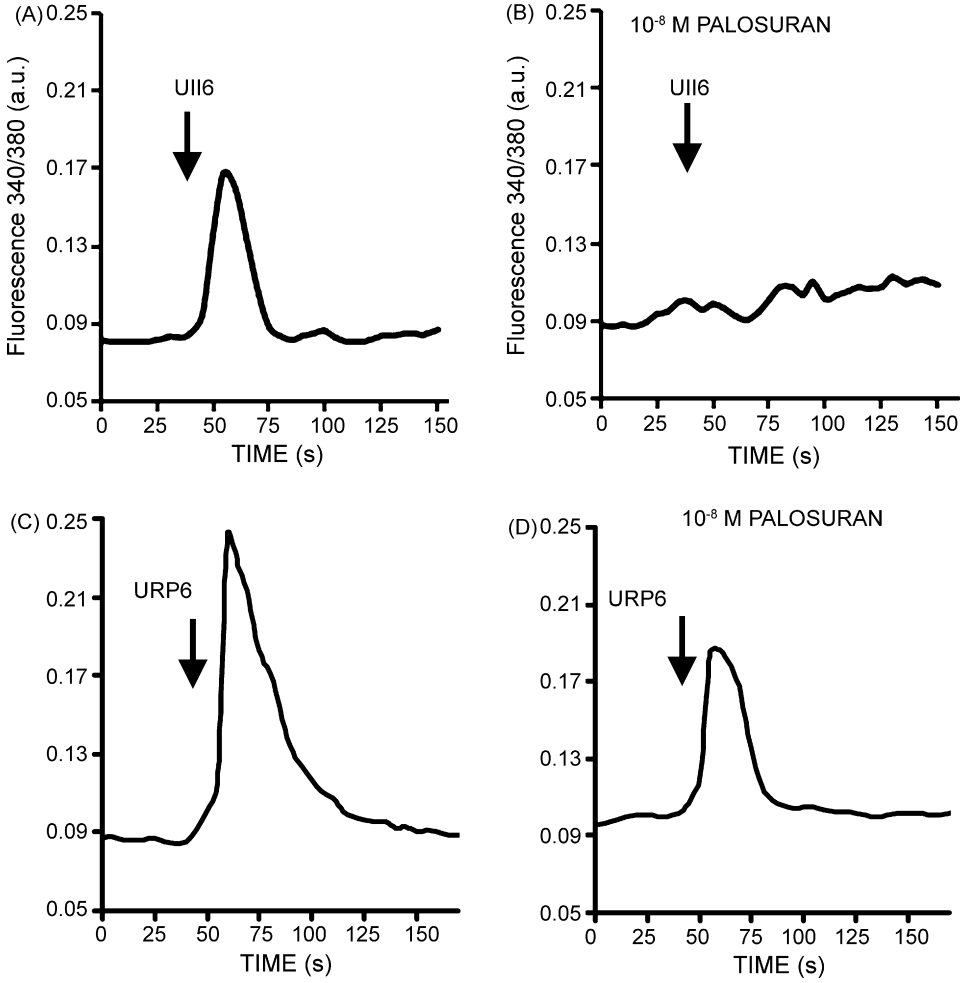

Fig. 3 – Effect of UII and URP on intracellular Ca^{2+} mobilization in stably transfected psst2-CHO-K1 cells. UII- and URP-induced increase of $[Ca^{2+}]_i$ in the absence (A and B, respectively) or presence of the UII-receptor antagonist, palosuran (C and D, respectively). In control conditions, UII and URP (10^{-6} M) significantly elevated $[Ca^{2+}]_i$ in psst2-expressing CHO-K1 cells. Palosuran (10^{-8} M) suppressed the effect of UII but did not inhibit that of URP on $[Ca^{2+}]_i$.

lower dose of UII was employed, administration of 10^{-7} M URP evoked a smaller $Ca^{2+}{}_i$ response than that induced by a higher dose of the peptide in terms of percentage of responsive cells (27.4%; $n = 28$ out of 98 cells) although not in that regarding the amplitude of the $[Ca^{2+}]_i$ increase ($261.3 \pm 18.4\%$ for 10^{-7} M URP). Finally, and in contrast to that found for UII, palosuran did not modify the percentage of cells responsive to 10^{-6} M URP (85.0%; $n = 102$ responsive cells out of 120 cells) and only slightly though significantly diminished maximal $[Ca^{2+}]_i$ increase induced by URP ($223.4 \pm 26.3\%$; $P < 0.05$; Fig. 3D).

3.4. *Measurement of $[Ca^{2+}]_i$ in stably transfected psst5 CHO-K1 cells in response to UII and URP*

The effects caused by UII and URP on $[Ca^{2+}]_i$ by specific activation of psst5 were also analyzed. Both 10^{-6} M UII and 10^{-6} M URP were able to elicit a significant increase of $[Ca^{2+}]_i$, the latter treatment being more effective ($165.3 \pm 20.9\%$ *versus* $239.8 \pm 49.3\%$ for UII and URP, respectively; $P < 0.05$; Fig. 4A and C). However, the percentage of psst5-expressing CHO cells responsive to UII (9.9%; $n = 21$ responsive cells out of 212 total cells) and URP (14.1%; $n = 28$ responsive cells out of 198 total cells) were considerably lower than those observed for psst2-expressing cells. At a 10^{-7} M concentration, URP was also more potent than UII both in terms of the maximal $[Ca^{2+}]_i$ reached ($224.8 \pm 33.3\%$ *versus* $170.2 \pm 15.6\%$ for URP and UII, respectively) and in the proportion of responsive cells (28% *versus* 14%).

As that observed for psst2-expressing cells, palosuran alone also enhanced $[Ca^{2+}]_i$ in stably transfected psst5 cells ($255.4 \pm 24.8\%$ above baseline in 26 out of 98 cells). Pre-incubation of psst5-expressing CHO-K1 cells with palosuran numerically but not significantly reduced the UII- ($137.1 \pm 6.7\%$; Fig. 4B) and URP-induced increases in $[Ca^{2+}]_i$ ($179.6 \pm 2.5\%$; Fig. 4D). Additionally, the presence of palosuran in the culture medium did not alter the proportion of URP-responsive cells (15%; $n = 18$ responsive cells out of 120 total cells) and only slightly reduced that of UII-responsive cells (6.7%; $n = 8$ responsive cells out of 120 total cells).

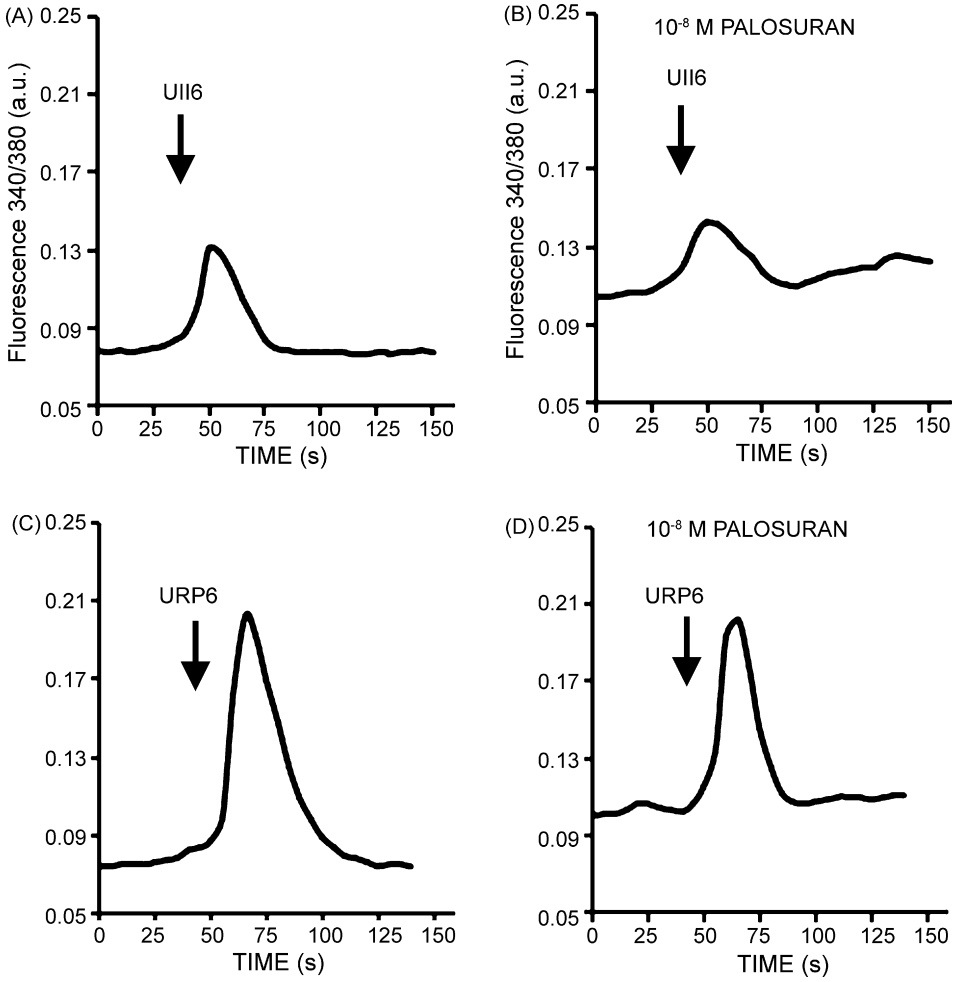

Fig. 4 – Effect of UII and URP on intracellular Ca^{2+} mobilization in stably transfected psst5-CHO-K1 cells. UII- and URP-induced increase of $[Ca^{2+}]_i$ in the absence (A and B, respectively) or presence of the UII-receptor antagonist, palosuran (C and D, respectively). In control conditions, UII and URP (10^{-6} M) significantly elevated $[Ca^{2+}]_i$ in psst5-expressing CHO-K1 cells. Palosuran (10^{-8} M) slightly reduced the amplitude of the response to UII (although differences did not reach statistical significance) but did not inhibit that of URP.

4. Discussion

In the present work, we have analyzed the effects elicited by UII and URP on cell proliferation and $[Ca^{2+}]_i$ through their action on specific somatostatin receptor subtypes, as compared to those triggered by somatostatin or by selective non-peptidic sst agonists. As CHO cells do not express any sst receptor subtype, they have been extensively used for the study of the specific effect of somatostatin and somatostatin-related compounds through their action on single, isolated receptor subtypes, as well as the internalization dynamics and oligomerization of the distinct ssts [38]. Likewise, CHO cells lack UTR expression [12] thus making these cells an excellent model to investigate the interaction between the somatostatin/sst and the UII/URP systems.

The results from the present study demonstrate that high doses of UII and URP are capable to specifically activate two somatostatin receptor subtypes, sst2 and sst5. In line with these findings, it has been suggested that type 2/5 somatostatin receptors and UTR display similar surface topologies, as somatostatin receptor antagonists with preferential affinity for hsst2 and hsst5 dose-dependently inhibit human UII-induced Ca^{2+} transients in rat thoracic aorta rings [45]. Accordingly, our results show that administration of UII inhibited cell growth in stably transfected psst5 CHO-K1 cells. In contrast to that found for UII, its inactive analog $[Ser^{5,10}]hUII_{4-11}$ [23] had no effect on cell proliferation. On the other hand, similar to that observed for UII, both somatostatin and the selective sst5 agonist L-817,818 also elicited an inhibitory action on cell growth in psst5-expressing cells, thus supporting the view that the porcine receptor, as that from other species [7], could mediate antiproliferative effects. Nevertheless, UII was found to be much less potent than the psst5-specific ligands in inhibiting cell growth. Intriguingly, the effect of L-817,818 on cell growth in psst5-expressing cells was observed at a wide range of doses of the agonist, which might be related to the ability of psst5 to form homodimers as well as higher ordered structures (i.e. oligomers) (our unpublished results). Future studies will be aimed at elucidating the dimerization properties of psst5 in response to the sst family of ligands.

Interestingly, and in contrast to that found for UII, URP had no significant effect on cell proliferation in cells expressing sst5, which points out the relevance of the N-terminal region of UII for this peptide to regulate cell growth through activation of sst5. Notwithstanding this, data on intracellular Ca^{2+} measurement suggest that both UII and URP, in pharmacological doses, are likely recognized by sst5, as the two peptides induced significant, though modest changes in $[Ca^{2+}]_i$ in psst5-expressing cells.

Contrary to that found for psst5, UII action through psst2 resulted in the stimulation of cell proliferation in CHO-K1 cells. Moreover, the peptide also increased $[Ca^{2+}]_i$ in psst2-transfected cells. In line with these findings, a previous report by Nothacker et al. [37] showed that human UII was able to mobilize $[Ca^{2+}]_i$ in rat sst2-expressing CHO cells, albeit only at very high concentrations. In the present study, we have also shown that URP was able to elicit cell growth and $[Ca^{2+}]_i$ mobilization in psst2-expressing CHO-K1 cells in a manner comparable to that observed for UII. Indeed, both UII and URP induced higher $[Ca^{2+}]_i$ increases and in a higher proportion of cells through activation of psst2 than via psst5. This might be due to differences in the receptor density expressed by the transfected cell lines or, alternatively, these results may indicate that psst2 is less selective than psst5 in recognizing the members of the UII family. Nevertheless, and irrespectively of the opposite effects mediated by psst2 and psst5 on cell proliferation and of their distinct efficiency to increase $[Ca^{2+}]_i$, our results suggest that the differential action of UII and URP on cell proliferation depends on the sst receptor subtype expressed rather than on the nature of the treatment, as somatostatin and selective somatostatin agonists mimic the inhibitory (psst5) and stimulatory effect (psst2) on growth of CHO cells. In this context, it is worth noting that in cultured pig pituitary cells, selective activation of sst2 and sst5 also evokes opposite responses on growth hormone (GH) secretion [30]. To be more specific, the sst2 agonist L-779,976 inhibited GH release whereas the sst5 agonist L-817,818 evoked a stimulatory effect on hormone secretion in porcine pituitary cell cultures [30]. Together, these findings support the view that, as shown previously for sst2 and sst5 from other species [17], the two porcine somatostatin receptors are coupled to distinct intracellular signalling pathways which, in turn, may mediate divergent cellular responses depending on the cell type.

The ability of psst2 to mediate a mitogenic response appears to be an intrinsic feature of this receptor as it was also observed in response to its natural ligand, somatostatin, as well as to the selective sst2 agonist L-779,976. These latter results were somewhat unexpected, as sst2 has been classically considered as a negative cell growth regulator (reviewed in Refs. [17,29]). In fact, expression of the mouse, rat or human sst2 in CHO cells inhibits mitogenic signals of serum or growth factors [2,3,7,17]. This species variation in properties of sst2 might be explained by divergences in the amino acid sequences which, in turn, may be responsible for their differences in G protein association and ability to trigger various signalling pathways in CHO cells. In support of this notion, previous studies have shown that psst2 is less efficient than its human counterpart in inhibiting forskolin-induced cAMP production in CHO cells [16]. Interestingly, it has been reported that the rat sst2(b) splice variant, which differs from the sst2(a) isoform in the length and composition of its intracellular C-terminus, mediates somatostatin-induced stimulation of cell proliferation when recombinantly expressed in CHO-K1 cells [2], further supporting the view that differences in receptor sequence may underlie the opposite response mediated by the distinct sst2 on cell proliferation.

As mentioned earlier, somatostatin exhibited lower potency than the sst5 agonist L-817,818 in the cell proliferation assays, an observation that was also observed, although in a lesser extent, with respect to the sst2 agonist L-779,976 in psst2-expressing cells. These results were somehow unexpected as previous results from our group have shown that somatostatin bound to psst2 in transfected CHO-K1 cells with only a slightly lower affinity than L-779,976 (EC_{50} values of 2.10 ± 0.42 nM and 1.12 ± 0.12 for somatostatin and L,779,976, respectively) [16]. Likewise, L-779,976 and L-817,818 exhibit K_i values similar or slightly lower than the natural ligand somatostatin in CHO-K1 cells expressing human sst2 and

sst5, respectively [43,44]. In line with these results, both compounds inhibited cAMP accumulation to the same extent as somatostatin in CHO-K1 transfected cells [43,44]. However, L-779,976 was approximately 10 times more potent than somatostatin for inducing cAMP inhibition in CHO cells transfected with rat sst2 [27]. Though it is still unknown whether the nonpeptide sst agonists elicit similar effects on cell proliferation than somatostatin through activation of ssts in other species, it is reasonable to propose that the relative potency and/or efficacy of the distinct sst ligands to induce particular cellular responses may differ among species.

Due to the multiple and relevant physiological roles suggested for UII (and, for that matter, also for URP), the development of UTR agonists and antagonists has received much attention during the last years [9,12,18,24–26,40]. Among the latter, the nonpeptide compound palosuran (ACT-058362) has been shown to act as a potent and specific antagonist of the human UTR [9]. Indeed, palosuran binds UTR with high affinity and potently displaces UII from binding to its receptor [9]. In addition, palosuran appears to antagonize not only the action of UII but also that of URP at the UTR, as this compound has been recently shown to inhibit reductions in both perfusion pressure and myocardial damage marker release induced by UII or URP in isolated perfused rat heart [42]. In line with these findings, in the present study we have found that palosuran antagonized the effect of UII on cell proliferation and $[Ca^{2+}]_i$ in psst2-transfected CHO-K1 cells. However, palosuran behaved as an agonist at psst5 as, similar to that found for somatostatin, the sst5 agonist L-817,818 or UII, it decreased basal proliferation in psst5-expressing cells. Moreover, palosuran-induced effect on cell growth was counteracted by UII, which by itself did not modify the proliferation rate of psst5-transfected CHO-K1 cells when administered at a 10^{-6} M dose, thus indicating that UII antagonizes the action of palosuran at ppst5. When viewed together, our present data strongly suggest that palosuran and UII compete for binding to the different ssts, making apparent the structural and functional relationships between ssts and UTR. Indeed, UTR shares with the different ssts the motif YANSCANP within the seventh transmembrane domain, which has been considered as a mammalian somatostatin receptor signature [38]. Interestingly, and contrarily to that shown for UII, palosuran was unable to inhibit the action of URP on psst2- or psst5-expressing cells, thus demonstrating the selective and specific antagonism of this compound on UII activity, at least at psst2. As mentioned before, proliferation studies showed that palosuran possesses intrinsic activity at psst5. Additionally, this drug also enhanced $[Ca^{2+}]_i$ when administered alone in both psst2- and psst5-transfected CHO-K1 cells thus supporting the view that this compound acts as a psst2 and psst5 partial agonist. On the other hand, a recent report by Marco and coworkers [32] has shown that palosuran has no effect on basal insulin release in perfused rat pancreas, a tissue which expresses both sst2 [21] and sst5 [34]. Although species-specific differences may exist in the sensitivity of these receptors to palosuran and the relative efficacy of this drug may differ depending on the response being measured, as suggested previously for certain ligands acting at GPCRs (reviewed in Ref. [19]), our results suggest that some caution must be taken when interpreting the data on the effects of this compound, at least on those tissues endogenously expressing sst2 and/or sst5.

In summary, the present results demonstrate that, similar to that found for somatostatin, both UII and URP are able to activate sst2 and sst5 in transfected CHO-K1 cells. These data, together with the observation that, reciprocally, somatostatin and cortistatin can bind and activate UTR [8,28], are in agreement with the notion of a common phylogenetic origin of the somatostatin/cortistatin and the UII/URP systems.

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