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Short communication

Urantide mimics urotensin-II induced calcium release in cells expressing recombinant UT receptors

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Abstract

Urotensin-II is the natural ligand of the UT receptor. This novel system is involved in the regulation of cardiovascular functions. Recently, a urotensin-II analog ([Pen^5,DTrp^7,Orn^8]urotensin-II(4–11)) named urantide, has been proposed as a selective and potent UT receptor antagonist. In order to pharmacologically characterize this new compound, urantide was tested on the native UT receptors of the rat aorta and on the human recombinant receptors expressed in CHO cells (CHO_{hUT}). Indeed, urantide behaves as a competitive, potent (pA_2 8.24), and pure antagonist in the rat aorta bioassay, while as an agonist (pEC_{50} 8.11) in a calcium mobilization assay performed in CHO_{hUT} cells. Urantide should be considered a low efficacy partial agonist.

Keywords: Urotensin-II; Urantide; UT receptor; Ca^{2+}; CHO cell; Aorta, rat

1. Introduction

Urotensin-II is a cyclic undecapeptide originally isolated from goby fish urophysis (Bern and Lederis, 1969). Five years ago, urotensin-II was identified as the natural ligand of an orphan G-protein coupled receptor (Ames et al., 1999) now referred to as UT receptor (Douglas and Ohlstein, 2000). The urotensin-II/UT receptor system seems to play an important role in cardiovascular functions (Douglas et al., 2004) although central nervous effects of urotensin-II have also been described (Gartlon et al., 2001; Matsumoto et al., 2004). Specific and selective UT receptor antagonists should provide useful tools for investigating the biological role(s) of the urotensin-II/UT receptor system.

Structure–activity studies revealed that the cyclic portion (Cys^5–Cys^{10}) of the peptide is crucial for biological activities, and the sequence Trp^7–Lys^8–Tyr^9 has been shown to be the most important for UT receptor occupation and activation (Brkovic et al., 2003; Flohr et al., 2002). The replacement of Lys^8 with Orn^8 lead to the identification of the low potency UT receptor partial agonist [Orn^8]urotensin-II (Camarda et al., 2002). Grieco et al. (2002) replaced Cys^5 with penicillamine in the octapeptide urotensin-II(4–11), thus generating [Pen^5]urotensin-II(4–11) that behaved as a potent UT receptor agonist. Recently, these chemical modifications were combined with Trp^7→DTrp^7 substitution, leading to identification of [Pen^5,DTrp^7,Orn^8]urotensin-II(4–11) (urantide). This peptide binds with high affinity to the

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human recombinant UT receptor and behaves as a pure, potent, and competitive UT receptor antagonist in the rat aorta bioassay (Patacchini et al., 2003).

In the present study, in order to characterize the pharmacological features of urantide, its actions were carefully reassessed in the rat aorta bioassay and compared in a calcium mobilization assay performed on CHO cells stably expressing the human UT receptor (CHO_{hUT}).

2. Material and methods

2.1. Rat aorta bioassay

All experimental protocols were approved by the ethics committee of the University of Ferrara. Male Sprague–Dawley rats (250–300 g; from Morini, Reggio Emilia, Italy) were decapitated under anaesthesia and the thoracic aortae were isolated and cut in helicoidal strips. The endothelium was rubbed and tissues were placed in organ baths containing Krebs solution at 37 °C and pH 7.4. A tension of 1 g was applied to the tissues and after 1 hour of stabilization, 1 μM noradrenaline (used as standard stimulus) was added to organ baths to assess tissue responsiveness. Cumulative concentration response curves to urotensin-II were performed in absence and in presence of urantide, incubated 30 min before at 10, 100 and 1000 nM. To assess the residual agonistic activity, higher concentrations (10 μM) of urantide were left in contact with the tissue for 30 min. Isometric tension was measured by a force transducer (GRASS FT03), and recorded using a multichannel polygraph (LINSEIS L2005).

2.2. Calcium levels in CHO_{hUT} cells

CHO_{hUT} were cultured in Dulbecco's Modification of Eagle's Medium Ham's F12 (50/50) supplemented with 10% foetal calf serum, 100 IU/ml penicillin and 100 μg/ml streptomycin and maintained at 37 °C in 5% CO_2 humidified air. Stock cultures were additionally supplemented with 800 μg/ml G418. Experimental cultures (G418 free) were harvested using ethylenediamine tetraacetic acid 1.7 mM, NaCl 154 mM and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) 10 mM buffer pH 7.4 and washed twice into Krebs/HEPES buffer (pH 7.4).

Intracellular calcium concentrations ($[Ca^{2+}]_i$) were measured in a cuvette based system (Perkin-Elmer LS50B fluorimeter) as described by Hirst et al. (1999) with slight modifications. Briefly, freshly harvested CHO_{hUT} cells were incubated with acetoxymethyl ester derivative of Fura 2, (fura 2-AM) (5 μM), for 30 min at 37 °C, followed by 20 min at room temperature to allow for complete dye de-esterification. $[Ca^{2+}]_i$ was calculated according to Grynkiewicz et al. (1985). Data are presented as change (Δ) in $[Ca^{2+}]_i$ (peak–prestimulus concentration).

2.3. Analysis of data

Data were analyzed by nonlinear curve fitting equation, using Graph Pad 3.0 software. Data are the mean±S.E.M. of *n* experiments and they were statistically analyzed using one way analysis of variance followed by the Dunnett test for multiple comparisons (Fig. 1) or the Student's *t*-test for unpaired data (Fig. 2).

2.4. Drugs and reagents

Urantide and urotensin-II were synthesized and purified at the Department of Pharmaceutical Sciences,

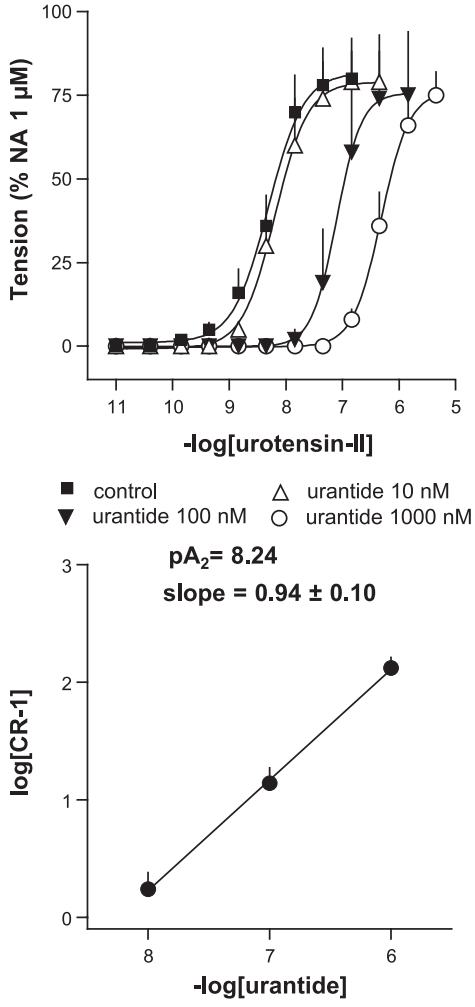

Fig. 1. Concentration–response curve to urotensin-II obtained in the absence (control) and in presence of increasing concentrations of urantide (10, 100, and 1000 nM) in the rat thoracic aorta (top panel). The corresponding Schild plot is shown in the bottom panel. Data are mean±S.E.M. of five experiments.

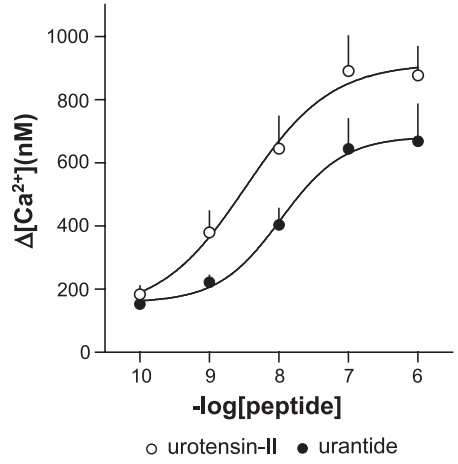

Fig. 2. Concentration–response curve to urotensin-II and urantide in CHO cells expressing human UT receptor. Effects are measured as change (Δ) in intracellular calcium concentration. Data are mean±S.E.M. of six experiments.

University of Ferrara. Tissue culture media and supplements were from Invitrogen (Paisley, Scotland). The other substances and reagents were purchased by Sigma (St. Louis, USA).

3. Results

3.1. Urantide antagonizes the contractile effects of urotensin-II in the rat thoracic aorta

As previously reported, urotensin-II was able to contract thoracic aortic strips in a concentration-dependent manner with high potency (pEC_{50} 8.31±0.11). Urantide, up to 10 μM, did not evoke any contractile effect. However, at 10, 100 and 1000 nM, urantide produced a concentration dependent and parallel rightward shift of the concentration–response curve to urotensin-II, without modifying its maximal effects (Fig. 1, top panel). Schild analysis of the data is compatible with a competitive type of antagonism (slope 0.94±0.10 not significantly different from unity) and yielded a pA_2 value of 8.24 (Fig. 1, bottom panel).

3.2. Urantide mimics the $[Ca^{2+}]_i$ release induced by urotensin-II in CHO_{hUT} cells

In CHO_{hUT} cells, urotensin-II produced a concentration-dependent increase in $[Ca^{2+}]_i$ displaying high potency (pEC_{50} 8.57±0.27). This stimulatory action of urotensin-II was mimicked by urantide that showed slightly lower potency (pEC_{50} 8.11±0.15) and maximal effect. The difference in maximal effects elicited by urotensin-II and urantide (E_{max} 917±87 and 726±147 nM, respectively) did not reach statistical significance (Fig. 2).

4. Discussion

Urantide has been recently reported to be a potent ligand of the UT receptor (Patacchini et al., 2003). This peptide binds with nanomolar affinity (pK_i 8.3) to the recombinant hUT and antagonizes (pK_B 8.3) the contractile effects of urotensin-II in the rat aorta without showing any residual agonist activity (Patacchini et al., 2003). Our findings confirm these data. Indeed, urantide (i) binds with similar high affinity (pK_i 7.9–9.0) to mouse, rat, and human UT receptors (Douglas, unpublished results), (ii) behaves as a pure antagonist in the rat aorta with high potency (pA_2 8.2) similar to that previously reported, (iii) it does not show any residual agonist activity up to micromolar concentrations in the rat aorta. Therefore, urantide represents an interesting UT receptor ligand which may be of use in future studies aimed to investigate the biological roles played by the urotensin-II/UT receptor system. However, urantide mimicked the effects of urotensin-II on $[Ca^{2+}]_i$ release in CHO_{hUT} cells thus acting as a UT receptor agonist. In this assay, the peptide displayed a potency value threefold lower than that of urotensin-II and a maximal effect equal to ~80% of that induced by the natural peptide. The different pharmacological behaviour of urantide [pure antagonist in the rat aorta vs. (high efficacy partial) agonist in CHO_{hUT} cells] could be attributed to species-specific differences between rat and human UT receptors. Although this possibility cannot be ruled out, some lines of evidence make it unlikely. First, urantide is a urotensin-II related peptide and urotensin-II (as is common for naturally occurring peptides) does not discriminate between species related receptor homologues (Douglas et al., 2004). Second, binding data performed in one of our laboratories (SAD) suggests that urantide displays a similar affinity at mouse, rat and human UT receptors. Third, urantide contains the Orn^8 substitution that was applied to the urotensin-II sequence and produced a reduction but not a complete elimination of ligand efficacy. In fact, [Orn^8]urotensin-II behaves mainly as an antagonist in the rat aorta bioassay (Camarda et al., 2002) and in vivo in mice against urotensin-II induced plasma extravasation (Vergura et al., 2003), as a typical partial agonist (intrinsic activity ≈0.5) in the inositol-phosphate formation assay and as a full agonist in $[Ca^{2+}]_i$ mobilization assay performed on cells expressing the hUT (Camarda, 2004). In addition, [Orn^8]urotensin-II behaves as a full UT receptor agonist also in the $[Ca^{2+}]_i$ release assay performed on cells expressing the rat recombinant receptor (Camarda et al., 2002). Collectively, we feel that the agonist/antagonist behaviour of urantide should be attributed to the different efficiency of the stimulus–response coupling which characterizes the rat aorta and $[Ca^{2+}]_i/CHO_{hUT}$ assays, being low for the former and very high for the latter. This difference is well recognized to influence the evaluation of ligand efficacy (Kenakin,

2002). Thus, the efficacy of urantide (and [Orn^8]urotensin-II) is overestimated in the cell system expressing high levels of recombinant receptors (Camarda et al., 2002), while in the rat aorta, where the density of UT sites is very low (Ames et al., 1999), the efficacy of [Orn^8]urotensin-II is underestimated and that of urantide cannot be detected. Further studies performed using inducible receptor expression cell systems (McDonald et al., 2003) are under way for validating this hypothesis.

In conclusion, the present results confirm pure antagonist behaviour of urantide in the rat aorta assay while revealing agonist activity of this UT receptor ligand in the $[Ca^{2+}]_i$ release assay performed in CHO_{hUT} cells. Residual agonist activity may limit the usefulness of urantide for pharmacological and physiopathological studies on the urotensin-II/UT receptor system. Nevertheless, urantide represents the most interesting peptide ligand for the UT receptor reported to date and will be useful as a template for future SAR studies aimed to the identification of selective, potent, and pure antagonists for the UT receptor.

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