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Human urotensin II-induced aorta ring contractions are mediated by protein kinase C, tyrosine kinases and Rho-kinase: inhibition by somatostatin receptor antagonists

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Abstract

Human urotensin II-(1–11) and its N-terminally shortened analogues, human urotensin II-(4–11)-OH and human urotensin II-(4–11)-NH_2 are potent vasoconstrictor peptides in isolated rat thoracic aorta. Human urotensin II-induced tonic aorta ring contractions are inhibited by the Ca^{2+} channel antagonists, verapamil, nitrendipine and diltiazem; D609 (Tricyclodecan-9-yl-xanthogenate, K), selective inhibitor of phosphatidylcholine-specific phospholipase C and partially by phospholipase C inhibitor U-73122 {1-[6-((17ß-3 Methoxyestra-1,3,5(10)-trien-17-yl)amino)hexyl]-1*H*-pyrrole-25-dione} and a selective inhibitor of phosphatidyl-inositol-specific phospholipase C-ET-18-OCH3 (Edelfosine,1-*O*-octadecyl-2*O*-methyl-rac-glycero-3-phosphorylcholine); protein kinase C inhibitors, chelerythrine and NPC-15437 {*S*-2,6-diamino-*N*-[[1-(1-oxotridecyl)-2-piperidinyl]methyl]-hexanamide dihydrochloride}; tyrosine kinase inhibitors, genistein and tyrphostin B42 and Rho-kinase inhibitor HA-1077 [1-(5-isoquinolinylsulfonyl)-homopiperazine dihydrochloride]. This indicates that human urotensin II-induced tonic contractions of the rat aorta are mediated by phospholipase C, protein kinase C, tyrosine kinases and Rho-kinase related pathways. In the high K^+ medium, human urotensin II induces dose-dependent phasic oscillations of aortic rings. These are inhibited by Ca^{2+} channel antagonists, the phospholipase C inhibitor, U-73122 and protein kinase C inhibitors, chelerythrine and NPC-15437, indicating that human urotensin II-induced phasic oscillations of the rat aorta are mediated by phospholipase C and protein kinase C-dependent pathways. Given their close structural similarity, several somatostatin analogues, importantly containing $DCys^5$ and $DTrp^7$ and expressing different degrees of somatostatin receptor antagonist activity, were tested for possible inhibitory effects on human urotensin II-induced contractions of the rat aorta rings. Pre-incubation of rat aorta rings in the presence of somatostatin analogues, which are preferentially sst_2 specific binders: PRL-2882; PRL-2903 and PRL-2915 at micro-molar concentrations significantly blocked the development of human urotensin II-induced tonic contractions. Somatostatin receptor antagonists dose-dependently inhibited human urotensin II-induced Ca^{2+} transients in rat thoracic aorta rings. These somatostatin receptor antagonists displayed moderate affinities for recombinant rat and human urotensin II receptor binding sites. The data support the suggestion that urotensin II receptor and somatostatin type 2/5 receptors display similar surface topologies and that analogues of somatostatin could provide useful lead compounds for the development of more potent urotensin II receptor antagonists.

Keywords: Urotensin II, human; Aorta contraction, rat; Phospholipase C; Protein kinase C; Tyrosine kinase; Rho-kinase; Somatostatin; Somatostatin-receptor antagonist

1. Introduction

Human urotensin II, the precursor sequence of which has been recently cloned (Coulouarn et al., 1998), is composed of 11 amino acid residues retaining a conserved cyclic hexapeptide and an N-terminal region consisting of four amino acid residues (Glu-Thr-Pro-Asp) which is highly variable across animal species. A high affinity rat receptor for urotensin II has been identified as the GPR14 orphan G protein-coupled receptor which has much similarity to the somatostatin and opioid receptor family and has been designated as UII-R1a (Liu et al., 1999). Simultaneously, a human G-protein-coupled receptor that has 75% sequence similarity to the orphan rat receptor GPR14 and exhibits the

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pharmacological characteristics of a human urotensin II receptor was also cloned (Ames et al., 1999). Human urotensin II binds with high affinity to this receptor, and the binding is functionally coupled to intracellular calcium mobilization. Human urotensin II and its prepro-mRNA have been localized primarily in the central nervous system and cardiovascular system and in smaller amounts in other organs (Coulouarn et al., 1998, 1999; Ames et al., 1999; Maguire et al., 2000).

Urotensin II was found to be the most potent of the known vasoactive peptides, although its activity is generally restricted to the arterial part of the vasculature and also varies depending on anatomical localization of these arteries (Ames et al., 1999). In isolated rat thoracic aorta fragments, urotensin II-induced contraction consisted of two distinct tonic and phasic components (Gibson, 1987) and it was suggested that the phasic component of contractions resulted from the movement of free extracellular Ca^{2+} through the nitrendipine-sensitive voltage operated channels (Gibson, 1987; Gibson et al., 1988). The tonic component of the contractile response to urotensin II might involve influx of extracellular calcium and the activation of protein kinase C (Gibson et al., 1988) pathways. In rabbit aorta slices, human urotensin II increased the levels of [^{3}H]inositol phosphates in a concentration-dependent manner and with the same potency that it causes vasoconstriction (Opgaard et al., 2000). It was recently reported that human urotensin II-induced contractions of aorta smooth muscle cells are mediated by activation of a small GTPase Rho A and Rho-kinase-dependent pathways (Sauzeau et al., 2001). Human urotensin II also induced actin stress fiber formation and arterial smooth muscle cell proliferation (Sauzeau et al., 2001). No precise physiological functions of urotensin II in cardiovascular regulatory mechanism have been presented yet. However, it was previously found that *Gillichthys* urotensin II reduced blood pressure in anesthetized rats (Gibson et al., 1988) and in isolated rat heart it elicited a biphasic response on coronary flow, consisting of initial decreases of flow followed by sustained vasodilatation (Katano et al., 2000). Human urotensin II was also a potent endothelium-dependent relaxant in intact rat mesenteric arteries pre-contracted with methoxamine (Bottrill et al., 2000) and in human small pulmonary and systemic abdominal resistance arteries (Stirrat et al., 2001). Liu et al. (1999) have found that the orphan-G-protein-coupled receptor GPR14 has much sequence homology to members of the somatostatin receptor family and could be activated by somatostatin and cortistatin at micro-molar doses. Somatostatin receptors mediate multiple signal-transduction pathways, including the stimulation of Ca^{2+} mobilization and inositol phosphate production, the activation of protein tyrosine phosphatases and recently reported sst_2- and sst_3-mediated arachidonic acid mobilization (Alderton et al., 2001). In the pancreatic alpha-cells, somatostatin suppresses electrical activity through a Gi2-protein-dependent mechanism (Gromada et al., 2001).

The similar, multiple-transduction-pathways involvement of both peptides, as well as some similar functions in vascular smooth muscle physiology, justify the question of a possible role of somatostatin in the mechanisms of human urotensin II-induced vascular contractions. Better understanding of the mechanisms involved in the cooperation of these two potent peptide hormone families might be of value in cardiovascular physiology, pathology and pharmacology.

2. Materials and methods

2.1. Peptides

Human urotensin II-(1–11)-Glu-Thr-Pro-Asp-c[Cys-Phe-Trp-Lys-Tyr-Cys]-Val-OH; human urotensin II-(4–11)-OH; human urotensin II-(4–11)-NH_2; as well as somatostatin antagonist analogues: PRL-2814, 3-(2-naphthyl)Ala-D-(4-chlorophenyl)Ala-Tyr-D-Trp-Lys-Val-Phe-Thr-NH_2; PRL-2851, 3-(2-naphthyl)Ala-c[D-Cys-His-D-Trp-Lys-Val-Cys]-D-(3,3-di)Phe-NH_2; PRL-2882, (4-fluoro)Phe-c[D-Cys-(3-pyridyl)Ala-D-Trp-Lys-Val-Cys]-3-(2-naphthyl)Ala-NH_2; PRL-2903, (4-fluoro)Phe-c[D-Cys-(3-pyridyl)Ala-D-Trp-Lys-*tert*-Leu-Cys]-3-(2-naphthyl)Ala-NH_2; PRL-2915, (4-chloro)Phe-c[D-Cys-(3-pyridyl)Ala-D-Trp-Lys-*tert*-Leu-Cys]-3-(2-naphthyl)Ala-NH_2; PRL-2970, [(4-chloro)Phe-c[D-Cys-Tyr-D-Trp-Lys-Thr-Cys]-3-(2-naphthyl)Ala-NH_2; PRL-3155, (4-chloro)Phe-c[D-Cys-(4-amino)Phe-D-Trp-Lys-Thr-Cys]-3-(2-naphthyl)Ala-NH_2; PRL-3195, (4-chloro)-Phe-c[D-Cys-(3-pyridyl)Ala-D-Trp-NMeLys-Thr-Cys]-3-(2-naphthyl)Ala-NH_2 and PRL-3363, (4-chloro)Phe-c[D-Cys-(3-pyridyl)Ala-Trp-Lys-Thr-Cys]-(4-chloro)Phe-NH_2, were synthesized by standard solid phase methodologies on CS Bio (San Carlos, CA, USA) model CS 136 or Advanced ChemTech (Louisville, KY, USA) model 200 automatic peptide synthesizers and purified by reverse phase-high performance liquid chromatography (RP-HPLC) on C18 bonded silica gel columns (Dynamax-300A, 5 or 8 μm, 21.4 × 250 mm). Samples of the purified peptides were hydrolyzed in 4M methanesulphonic acid containing 0.2% 3-(2-aminoethyl)indole and subjected to amino acid analyses performed using an automatic HPLC system (Varian, Walnut Creek, CA, USA). Molecular weights were determined by matrix assisted laser desorption mass spectrometry using a LaserMat 2000 mass spectrometer (Finnegan MAT, San Jose, CA, USA) with substance P (1348.7 Da) as an internal standard. The somatostatin-receptor antagonists originated from the most potent our cyclic somatostatin analogue DC-38-48 {[*H*-3-(2-naphthyl)Ala^5-c[D-Cys-(3-pyridyl)Ala^7-D-Trp-Lys-Val^{10}-Cys]-3-(2-naphthyl)Ala^{12}-NH_2}. This group of somatostatin-analogue antagonists consists of peptides having different substitutions at positions: 5; 7; 10 and 12 of the parent peptide and all have D-Cys at position 6 and, D-Trp at position 8. Somatostatin analogues were tested by incorporation of the peptide (in freshly prepared solution) at concentrations ranging from 10^{-8} to 10^{-5} M into incubation

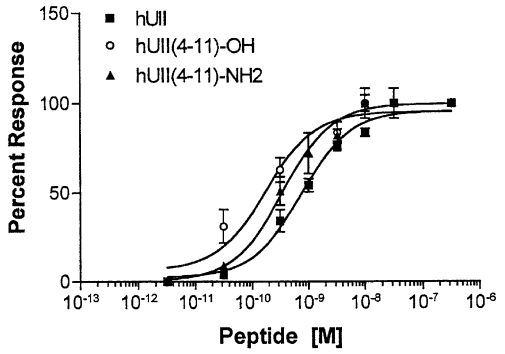

Fig. 1. Concentration-dependent constriction of isolated endothelium-preserved rat thoracic aorta rings by human urotensin II-(1–11)-OH (filled squares); human urotensin II-(4–11)-OH (open circles); and human urotensin II-(4–11)-NH_2 (filled triangles). Responses are shown relative to 100 mM KCl (accepted as 100%). Values are mean and vertical bars represent S.E.M. ± mean, $n = 3$.

medium 5–15 min before introduction of human urotensin II at concentrations of 1 or 3×10^{-9} M; or were added into incubation medium after human urotensin II-induced tonic contraction had reached plateau.

2.2. Chemicals

Ca^{2+} channel blocker nitrendipine; Ca^{2+} channel antagonists (L-type), (±)-verapamil hydrochloride and diltiazem hydrochloride; protein kinase C inhibitor, chelerythrine chloride and selective protein kinase C inhibitor, NPC-15437; tyrosine kinase inhibitor, tyrphostin B42; tyrosine kinase and topoisomerase II kinase inhibitor, genistein; phospholipase C inhibitor, U-73122 and RhoA-kinase inhibitor. HA-1077 (Farudil) were purchased from RBI-Sigma, Natick, MA, USA. ET-18-OCH3, an agent that selectively inhibits phosphatidylinositol-specific phospholipase C; and D609, selective inhibitor of phosphatidylcholine-specific phospholipase C, were purchased from Calbiochem, San Diego, CA. All other chemicals were purchased from Sigma, St. Louis, MO. [^{125}I]goby urotensin II was custom-iodinated by Dr. T.P. Davis, University of Arizona.

2.3. Animals and aorta ring contraction assays

Male Sprague–Dawley rats (250–350 g) were sacrificed by decapitation. The thoracic aorta was dissected and freed from connective tissue and cut into ~1.5 mm in width rings which were vertically suspended in 15 ml organ bath containing normal Krebs solution containing: 118 mM NaCl; 25 mM $NaHCO_3$; 4.7 mM KCl; 1.2 mM KH_2PO_4; 1.2 mM $MgSO_4$ (anhydrous); 15 mM $CaCl_2$ (dihydrate); 11 mM D-glucose, or in high potassium Krebs solution (NaCl was replaced by KCl). Optimal tension was applied (1 or 2 g for tonic and 0.2 g for phasic contractions) and the bath medium was maintained at 37 °C bubbled with a mixture of 95% O_2–5% CO_2. Some preparations, before being mounted in the organ bath, were rubbed with a moistened cotton wool swab in order to remove the endothelial cell layer and the effect of this procedure was tested using an acetylcholine-relaxation test. The aorta rings were allowed to equilibrate for 90 min at the optimal tensions prior to starting experiments. During the equilibration period, the bath solution was replaced every 15 min. At the end of the equilibration period, tissue contractions were initiated using 100 mM KCl solution. Changes in arterial smooth muscle tension were recorded isometrically using a force-displacement transducer (Radnoti) and recorded using AcqKnowledge ACK100 Version 3.2 (BIOPAC Systems, Santa Barbara, CA). After washing and tissue recovery, aorta rings were exposed to a single dose of freshly prepared peptide solution. Contractile responses of aorta rings in regular Krebs buffer were originally expressed in volts and finally calculated as the percentage of the contractile response to 100 mM KCl (accepted as 100%). In studies of human urotensin II-induced phasic oscillations (Ca^{2+} transients) in high potassium Krebs buffer we evaluated the effect of human urotensin II concentration as well as selected inhibitors on the transients amplitude using AcqKnowledge ACK100 Version 3.2 software. All animal used in the experiments were approved by the Advisory Committee for Animal Resources, Tulane University School of Medicine.

2.4. Receptor expression

The cDNAs of rat and human urotensin II receptors were cloned by polymerase chain reaction (PCR) using rat brain or human placenta cDNA (Clontech, Palo Alto, CA) respectively as a template. Gene specific primers flanking full-length coding sequence of rat urotensin II (S1: 5′-ATCCCAGTGTGAGGACCGAG-3′ and AS1: 5′-AGGATTGCACAGTGCACTCT-3′) and human urotensin

Table 1
In vitro inhibition of [^{125}I]urotensin II (goby) binding to transfected rat and human urotensin II receptors by goby, human urotensin II and human urotensin II-(4–11)-OH/NH_2 fragments and peptide stimulation of tonic contractions of rat aorta rings

Peptide	K_i (nM)		EC_{50} (nM)
	rU-II[a]	hU-II[b]	Rat aorta rings
Ala-Gly-Thr-Ala-Asp-c [Cys-Phe-Trp-Lys-Tyr-Cys]-Val-OH	2.41 ± 0.21	1.78 ± 0.18	–
Glu-Thr-Pro-Asp-c [Cys-Phe-Trp-Lys-Tyr-Cys]-Val-OH	2.34 ± 0.85	1.68 ± 0.31	0.73 ± 0.13
Asp-c[Cys-Phe-Trp-Lys-Tyr-Cys]-Val-OH	1.33 ± 0.29	1.20 ± 0.30	0.16 ± 0.10
Asp-c[Cys-Phe-Trp-Lys-Tyr-Cys]-Val-NH_2	1.02 ± 0.22	1.43 ± 0.29	0.37 ± 0.05

[a] Rat urotensin II.
[b] Human urotensin II.

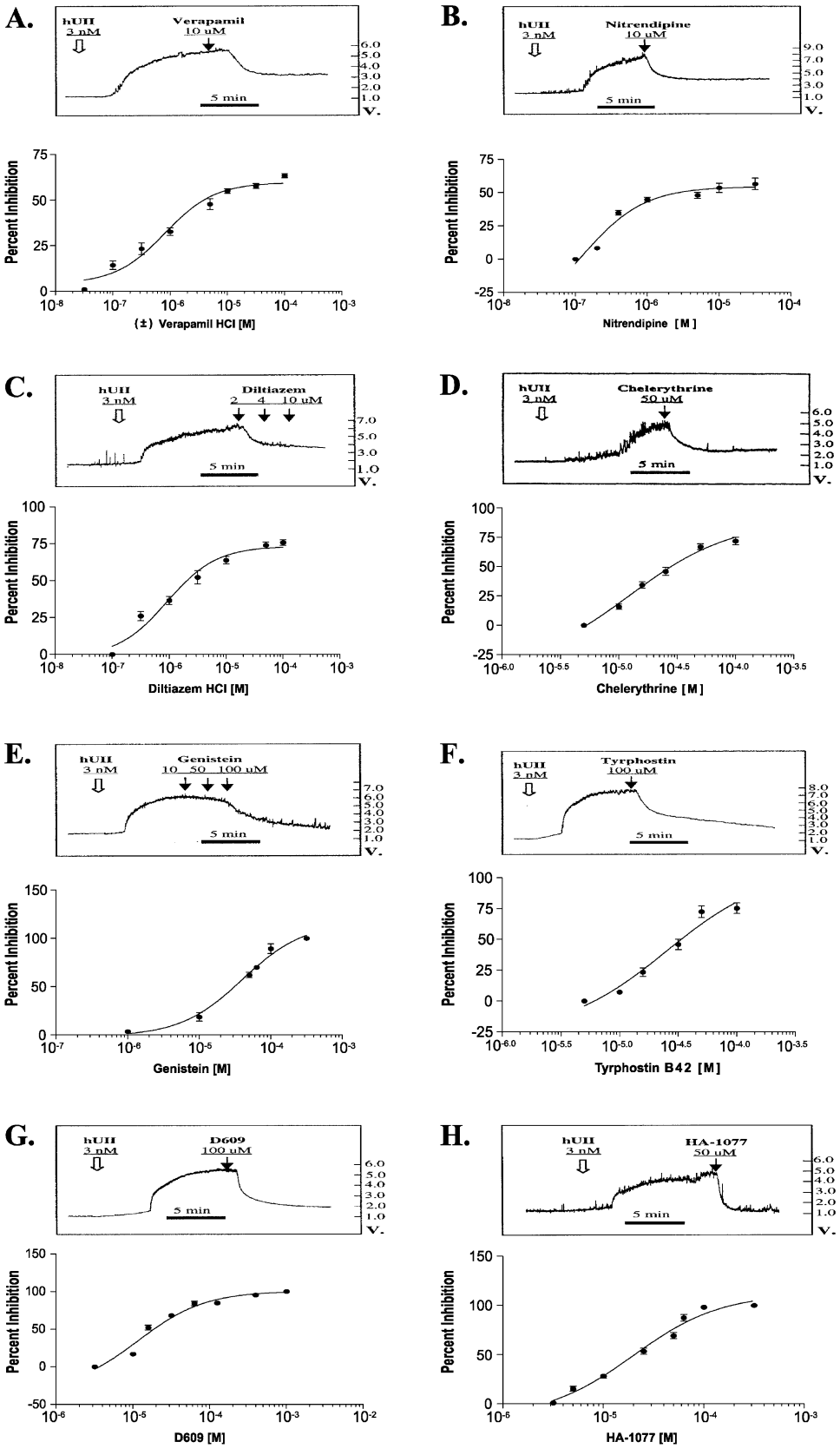
A.
hUII 3 nM
Verapamil 10 uM
5 min
V.
Percent Inhibition
(±) Verapamil HCl [M]
B.
hUII 3 nM
Nitrendipine 10 uM
5 min
V.
Percent Inhibition
Nitrendipine [M]
C.
hUII 3 nM
Diltiazem 2 4 10 uM
5 min
V.
Percent Inhibition
Diltiazem HCl [M]
D.
hUII 3 nM
Chelerythrine 50 uM
5 min
V.
Percent Inhibition
Chelerythrine [M]
E.
hUII 3 nM
Genistein 10 50 100 uM
5 min
V.
Percent Inhibition
Genistein [M]
F.
hUII 3 nM
Tyrphostin 100 uM
5 min
V.
Percent Inhibition
Tyrphostin B42 [M]
G.
hUII 3 nM
D609 100 uM
5 min
V.
Percent Inhibition
D609 [M]
H.
hUII 3 nM
HA-1077 50 uM
5 min
V.
Percent Inhibition
HA-1077 [M]

Table 2
Summary of effects of various inhibitors on human urotensin II-induced responses in rat thoracic aorta

Compound	Pharmacological action	Observed results	
		Krebs buffer	High K^+ Krebs buffer
(±)Verapamil	Ca^{2+} channel (L-type) antagonist	Inhibition (max. 63.4 ± 1.1%, $P<0.05$)	Inhibition complete at 3×10^{-6} M
Nitrendipine	Ca^{2+} channel blocker	Inhibition (max. 56.5 ± 4.3%, $P<0.05$)	Inhibition complete at 2×10^{-6} M
Diltiazem.HCl	Ca^{2+} channel (L-type) antagonist	Inhibition (max. 75.8 ± 1.9%, $P<0.05$)	Inhibition complete at 3×10^{-5} M
Chelerythrine	Protein kinase C inhibitor	Inhibition (max. 71.8 ± 3.2%, $P<0.05$)	Inhibition complete at 5×10^{-5} M
NPC-15437	Selective protein kinase C inhibitor	Inhibition (max. 75.9 ± 4.3%, $P<0.05$)	Inhibition complete at 10^{-5} M
U-73122	Phospholipase C and A2 inhibitor	Partial inhibition	Inhibition complete at 5×10^{-6} M
ET-18-OCH3	Selective PI phospholipase C inhibitor	Partial inhibition	n.d.
D609	Selective PC phospholipase C inhibitor	Inhibition complete at 10^{-3} M	n.d.
Genistein	Tyrosine kinase and topo-isomerase II inhibitor	Inhibition complete at 3×10^{-4} M	No effect
Daidzein	Inactive analog of genistein	No effect	No effect
Tyrphostin B42	Potent tyrosine kinase (selective Jak-2) inhibitor	Inhibition (max. 75.3 ± 4.3%, $P<0.05$)	No effect
HA-1077	Potent Rho-associated kinase inhibitor	Inhibition complete at 3 × 10 − 4 M	No effect

Data are means ± S.E.M.
PI = phosphatidyinositol; PC = phosphatidylcholine; n.d. = not determined.

II (S2: 5′ -CTCGTCTGGTGGCTCTTGAGTC-3′ and AS2: 5′ CATGTCTCCAGAAGCCGGGACTG-3′) and PCR optimization kit (Invitrogen, Carlsbad, CA) or GC-Rich PCR system (Roche Diagnostics, Mannheim, Germany) were used for PCR. The PCR product were cloned into pCR2.1 vector using Original TA Cloning Kit (Invitrogen). The full-length rat and human urotensin II cDNA were subcloned into mammalian expression vector pcDNA 3.1 (Invitrogen). The plasmids were transfected into Chinese hamster ovary cell line, CHO-K1 (American Type Culture Collection, Rockville, MD), by the calcium phosphate method. Single cell clones stably expressing the rat or the human urotensin II receptor were obtained by selecting transfected cells grown in cloning rings in RPMI 1640 media supplemented with 10% fetal bovine serum and 1 mM sodium pyruvate containing 0.8 mg/ml G418 (Gibco, Grand Island, NY).

2.5. Binding assay

Plasma membranes were prepared for radioligand binding studies by homogenization of the recombinant cells in 20 ml of ice-cold 50 mM Tris–HCl with a Brinkman Polytron (Westbury, NY) (setting 6, 15 s). The homogenates were washed twice by centrifugation (39,000 × *g*/10 min), and the final pellets were re-suspended in 50 mM Tris–HCl, containing 2.5 mM $MgCl_2$, 0.1 mg/ml bacitracin, and 0.1% bovine serum albumin (fraction V). For assay, aliquots (0.4 ml) were incubated with 0.05 nM [^{125}I]goby-urotensin II, with and without 0.05 nM of unlabeled competing test peptides. After a 45-min incubation (25 °C), the bound [^{125}I]goby-urotensin II was separated from the free by rapid filtration through GF/C filters (Brandel, Gaithersburg, MD), which had been previously soaked in 0.5% polyethyleneimine. The filters were then washed three times with 5-ml aliquots of ice-cold 50 mM Tris–HCl buffer containing 0.1% bovine serum albumin, and the bound radioactivity was counted by gamma spectrometry (Wallac LKB, Gaithersburg, MD). Specific binding was defined as the total [^{125}I]goby-urotensin II bound minus that bound in the presence of 1 μM goby urotensin.

2.6. Statistical calculations

Dose–effect curves were constructed from data obtained at different concentrations of the peptides. The percent of stimulation/inhibition by each tested peptide was calculated individually for each experiment. All values are expressed as mean ($n=3-8$) ± S.E.M. Statistical comparisons were made by analysis of variance (ANOVA) followed by Newman–Keuls procedures and differences were considered significant where $P<0.05$. EC_{50} and K_i values were calculated by means of nonlinear regression analysis of the concentration–response curves (after subtraction of a background values) using the GraphPad/Prism3 computer pro-

Fig. 2. Concentration-dependent effect of Ca^{2+} channel antagonists, verapamil, nitrendipine and diltiazem; protein kinase C inhibitor chelerythrine; tyrosine kinase inhibitors, genistein and tyrphostin B42; PLC inhibitor, D609; and Rho-kinase inhibitor, HA-1077, on human urotensin II-induced tonic aorta ring constrictions. Values are mean ± S.E.M., $n=3-5$. (A) Effect of verapamil on human urotensin II (3 nM) induced tonic aorta ring constrictions. (B) Effect of nitrendipine on human urotensin II (3 nM) induced tonic aorta ring constrictions. (C) Effect of diltiazem on human urotensin II (3 nM) induced tonic aorta ring constrictions. (D) Effect of chelerythrine on human urotensin II (3 nM) induced tonic aorta ring constriction. (E) Effect of genistein on human urotensin II (3 nM) induced tonic aorta ring constrictions. (F) Effect of tyrphostin B42 on human urotensin II (3 nM) induced tonic aorta ring constrictions. (G) Effect of D609, on human urotensin II (3 nM) induced tonic aorta ring constrictions. (H) Effect of HA-1077 on human urotensin II (3 nM) induced tonic aorta ring constrictions. Inserts represent selected original recordings of individual experiments. Open arrows indicate the addition of human urotensin II into the incubation vessel. Filled arrows indicate the additions of the respective agents into the incubation vessel. See text and Table 2 for average values. V = volts. Numbers on the right indicate the amplitude of the aorta ring constrictions expressed in volts.

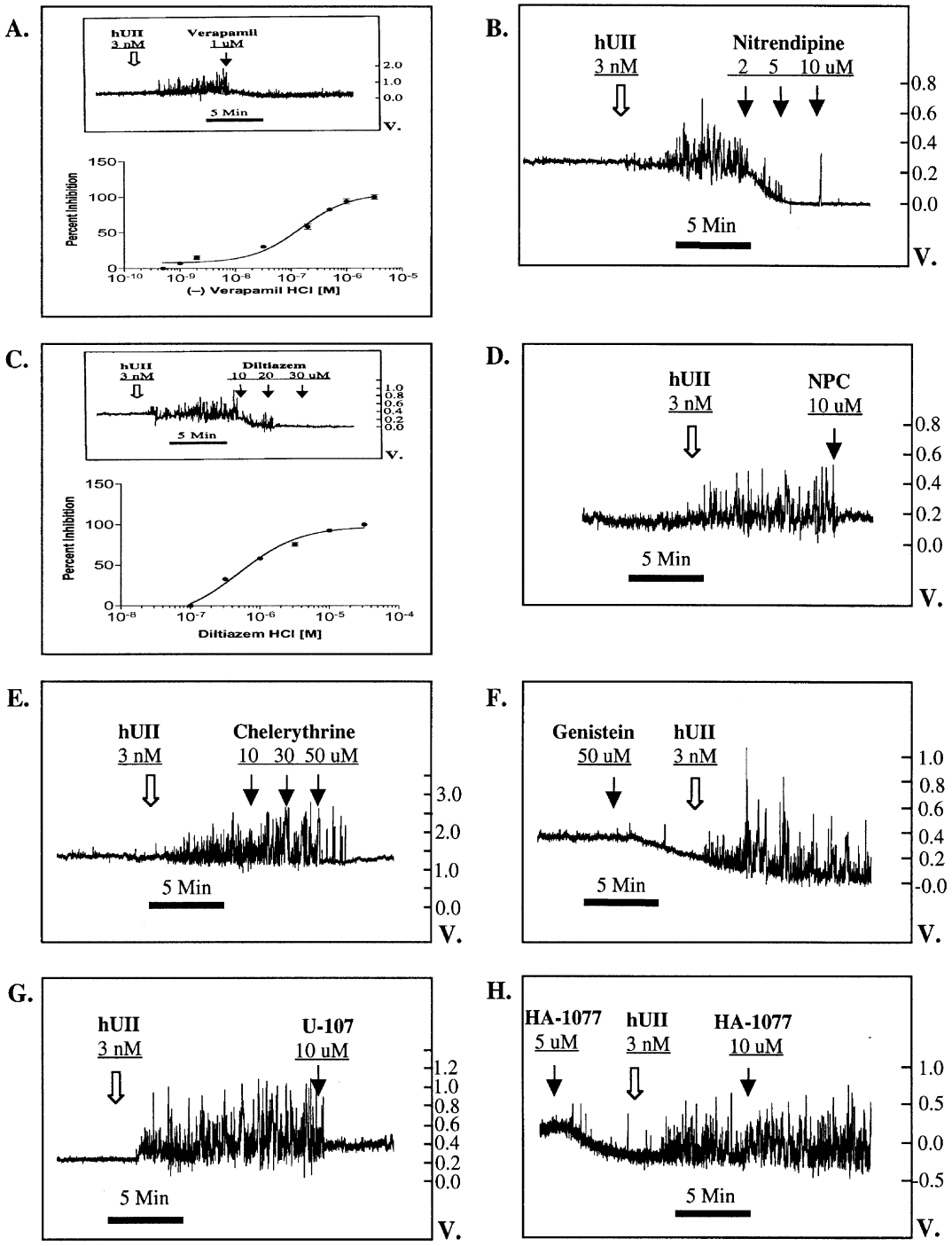

Fig. 3. Effect of Ca^{2+} channel antagonists, verapamil, nitrendipine and diltiazem; protein kinase C inhibitors, NPC-15437 and chelerythrine; tyrosine kinase inhibitor genistein; phospho-lipase C inhibitor, U-107; and Rho-kinase inhibitor, HA-1077 on human urotensin II induced phasic oscillations in the high potassium Krebs buffer. (A) Concentration-dependent effect of verapamil on human urotensin II (3 nM) induced phasic oscillations. In insert, effect of verapamil (1 μM) on phasic oscillations and a resting basal tension is shown. (B) Effect of nitrendipine (2, 5 and 10 μM) on human urotensin II (3 nM) induced phasic oscillations and a resting basal tension. (C) Concentration-dependent effect of diltiazem on human urotensin II (3 nM) induced phasic oscillations. In insert effect of diltiazem (10, 20 and 30 μM) on phasic oscillations and resting basal tension is shown. (D) Effect of NPC-15437 (10 μM) on human urotensin II (3 nM) induced phasic oscillations. (E) Effect of chelerythrine (10, 30 and 50 μM) on human urotensin II (3 nM) induced phasic oscillations. (F) Effect of genistein (50 μM) on human urotensin II (3 nM) induced phasic oscillations and a resting basal tension. (G) Effect of U-107 (U-73122), (10 μM) on human urotensin II (3 nM) induced phasic oscillations. (H) Effect of Rho-kinase inhibitor HA-1077 on human urotensin II (3 nM) induced phasic oscillations and a resting basal tension. HA-1077 (5 μM) was applied 5 min before human urotensin II and again (10 μM), 7.5 min after human urotensin II application. Open arrows indicate the addition of the human urotensin II into the incubation vessel. Filled arrows indicate the additions of the respective agents into the incubation vessel. See text for average values. All graphs represents a selected original experimental data. V = volts. Numbers on the right indicate the amplitude of the phasic oscillations expressed in volts.

Table 3
Comparison of binding affinities (K_i) of somatostatin analogues for cloned human somatostatin receptors (sst_1-sst_5) and rat and human urotensin II receptors and inhibition of human urotensin II-induced phasic oscillations of rat aorta (ED_{50})

Peptide	Sequence	K_i (nM)[a]					K_i (nM)		EC_{50} (nM)
		sst_1	sst_2	sst_3	sst_4	sst_5	rUII	hUII	Rat aorta rings
PRL-2814	Nal-D-Cpa-Tyr-D-Trp-Lys-Val-Phe-Thr-NH_2	>1000	706 ± 176	1130 ± 128	2840	1840 ± 772	6943 ± 251	1174 ± 63	4200 ± 820
PRL-2851	Nal-c[D-Cys-His-D-Trp-Lys-Val-Cys]-D-Dip-NH_2	1090 ± 87	18 ± 2	1200 ± 101	>1000	>1000	2485 ± 91	1348 ± 17	1330 ± 410
PRL-2882	4Fpa-c[D-Cys-3Pal-D-Trp-Lys-Val-Cys]-Nal-NH_2	484 ± 18	35 ± 8	271 ± 185	>1000	287	n.d.	n.d.	967 ± 260
PRL-2903	4Fpa-c[D-Cys-Pal-D-Trp-Lys-Tle-Cys]-Nal-NH_2	>1000	27 ± 3	231 ± 102	>1000	535 ± 116	310 ± 8	555 ± 54	4.3 ± 0.9
PRL-2915	4Cpa-c[D-Cys-3Pal-D-Trp-Lys-Tle-Cys-]Nal-NH_2	>1000	12 ± 1	100 ± 57	895	382 ± 58	293 ± 1	562 ± 27	589 ± 90
PRL-2970	Cpa-c[D-Cys-Tyr-D-Trp-Lys-Thr-Cys]-Nal-NH_2	>1000	26 ± 7	93 ± 5	>1000	48 ± 11	1424 ± 25	951 ± 14	45 ± 12
PRL-3155	Cpa-c[D-Cys-Apa-D-Trp-Lys-Thr-Cys]-Nal-NH_2	904 ± 96	10 ± 4	357 ± 218	n.d.	78 ± 2	1527 ± 138	1012 ± 136	23 ± 10
PRL-3195	Cpa-c[D-Cys-Pal-D-Trp-NMeLys-Thr-Cys]-Nal-NH_2	1000	17 ± 5	66 ± 6	1000	6 ± 1	429 ± 27	1846 ± 131	24 ± 12

rUII = rat urotensin II; hUII = human urotensin II; n.d. = not determined. Nal = 3-(2-naphthyl)alanine; Dip = 3,3-diphenylalanine; Pal = 3-pyridylalanine; Fpa = 4-fluorophenylalanine; Tle = *tert*-leucine; Cpa = 4-chlorophenylalanine; Apa = 4-aminophenylalanine.
[a] Data from Hocart et al. (1998, 1999).

gram (Dr. Harvey Motulsky, 1999. GraphPad Software). The equation chosen for fitting in the GraphPad program was: $Y = \text{Bottom} + (\text{Top} - \text{Bottom})/1 + 10^{\text{LogEC50}-X}$ where the variable Bottom is the Y value at the bottom plateau and Top is the Y value at the top plateau.

3. Results

Addition of human urotensin II-(1–11)-OH at doses of 0.3–10 nM to the rat aorta-rings incubated in regular Krebs medium produced slow developing tonic contractions lasting for over 30 min with an EC_{50} value of 0.73 ± 0.13 nM (Fig. 1 and Table 1). Elimination of the first three N-terminal amino acids (Glu-Thr-Pro) from human urotensin II, created a more potent analogue in this assay displaying an EC_{50} value of 0.16 ± 0.09 nM ($P < 0.02$) while the amidation of the C-terminus of the human urotensin II-(4–11) did not significantly affect its biological activity (Fig. 1 and Table 1). Both human and goby urotensin II inhibited binding of [^{125}I]UII (goby) to the urotensin II binding sites with similar K_i values of 1.68 ± 0.31 and 1.78 ± 0.18 nM, respectively (Table 1). The human urotensin II-induced tonic contraction component was dose- and time-dependently partially inhibited by the Ca^{2+} channel blocker verapamil with maximal inhibition of 63.4 ± 1.1% at a concentration of 100 μM (Fig. 2A); nitrendipine with maximal inhibition of 56.5 ± 4.3% at a concentration of 30 μM (Fig. 2B) and a selective L-type Ca^{2+} channel antagonist, dilthiazem, with a maximal inhibition of 75.8 ± 1.9% at a concentration of 100 μM (Fig. 2C; Table 2). Human urotensin II-induced rat aorta tonic contractions could be decreased by 71.8 ± 3.2% by the protein kinase C inhibitor, chelerythrine, at the concentration of 100 μM (Fig. 2D) and by the selective protein kinase C inhibitor, NPC-15437 (75.9 ± 4.3%). Genistein, tyrosine kinase and topoisomerase II kinase inhibitor, dose-dependently decreased tonic rat aorta ring contractions with full inhibition at concentration of 300 μM with an EC_{50} value of $3.9 \pm 0.4 \times 10^{-5}$ M (Fig. 2E) while daidzein (inactive analogue of genistein) at the dose of 300 μM was completely inactive. The specific tyrosine kinase inhibitor, tyrphostin B42, when applied at dose of 100 μM after human urotensin II-induced tonic aorta ring contractions had reached plateau, rapidly decreased tonic

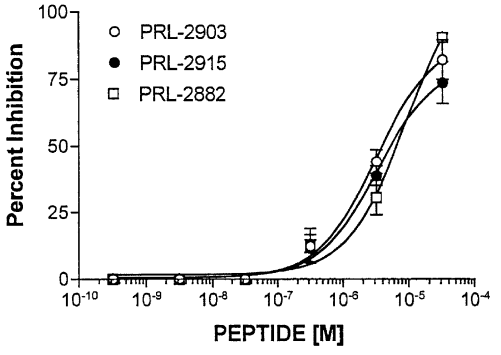

Fig. 4. Concentration-dependent effect of somatostatin receptor antagonists: PRL-2882 (open squares), PRL-2903 (open circles) and PRL-2915 (filled circles) on inhibition of human urotensin II (1 nM) induced rat aorta ring constrictions. Somatostatin receptor antagonists were added to the incubation vessels at concentrations of $3 \times 10^{-10}-3 \times 10^{-5}$ 15 min before human urotensin II (1 nM) and incubation was continued for an additional 15 min. Results are shown as the means ± S.E.M. of triplicate determinations.

contractions by 75.3 ± 4.3% (Fig. 2F). Pre-incubation of the rat aorta rings with tyrphostin B42 for 15 min dose-dependently inhibited human urotensin II-induced aorta ring contraction with complete inhibition of the tonic contraction occurring at 100 μM (EC_{50} value of $4.4 \pm 0.6 \times 10^{-5}$ M). ET-18-OCH_3 (Edelfosine), an agent that selectively inhibits phosphatidylinositol-specific phospholipase C, when pre-incubated (15 min) with aorta rings, dose-dependently partially inhibited human urotensin II-induced tonic contractions (maximal inhibition = 73.1 ± 1.4% at a concentration of

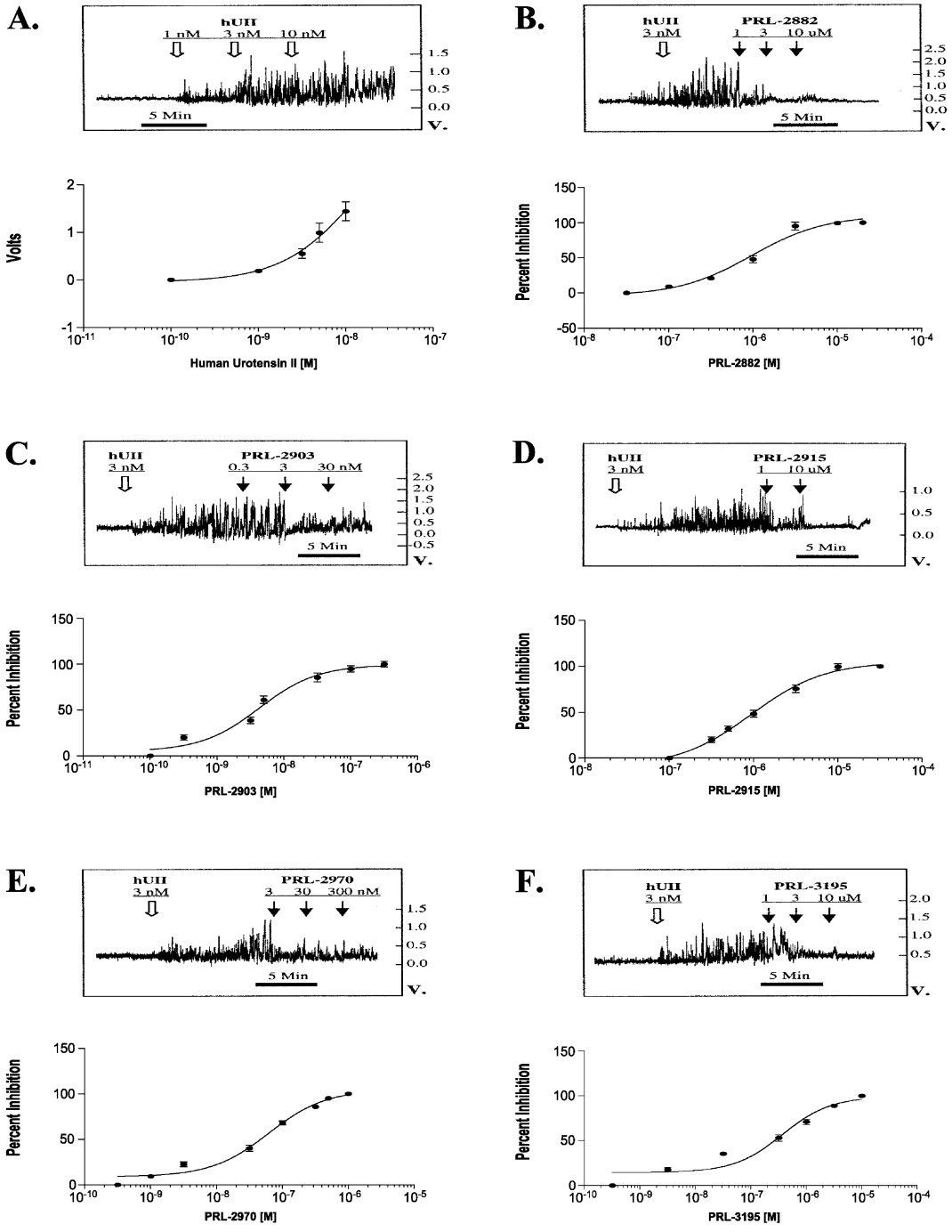

Fig. 5. Concentration-dependent effect of selected somatostatin receptor antagonists on human urotensin II-induced phasic oscillations of rat aortic rings in high potassium Krebs buffer. (A) Concentration-dependent human urotensin II-induced rat aortic ring phasic oscillations. (B) Effect of PRL-2882 (1, 3 and 10 μM on human urotensin II (3 nM) induced phasic oscillations. (C) Effect of PRL-2903 (0.3, 3 and 10 nM) on human urotensin II (3 nM) induced phasic oscillation. (D) Effect of PRL-2915 (0.3, 3 and 30 nM) on human urotensin II (3 nM) induced phasic oscillations. (E) Effect of PRL-2970 (3, 30 and 300 nM) on human urotensin II (3 nM) induced phasic oscillations. (F) Effect of PRL-3195 (1, 3 and 10 μM) human urotensin II (3 nM) induced phasic oscillations. Results are shown as the means ± S.E.M. of three to seven determinations. Inserts represent original tracings.

2.5×10^{-4} M), while D609, selective inhibitor of phosphatidylcholine-specific phospholipase C completely reversed human urotensin II-induced tonic aorta ring contraction ($EC_{50} = 1.2 \pm 0.2 \times 10^{-5}$ M) (Fig. 2G). HA-1077, a RhoA-kinase (ROK) inhibitor, completely reversed human urotensin II-induced tonic aorta ring contractions with an EC_{50} value of $2.0 \pm 0.1 \times 10^{-5}$ M, (Fig. 2H; Table 2), and when pre-incubated for 10 min in the presence of HA-1077 (100 μM) completely blocked human urotensin II-induced contractions. In the presence of high potassium Krebs buffer, human urotensin II generated phasic transients (Ca^{2+} transients, Ca^{2+} oscillations) or phasic oscillations of the rat aorta rings. Human urotensin II-induced phasic oscillations of the aorta rings were generated in a dose-dependent manner and displayed a dose-dependent stimulation of their amplitude (mean of the maximal amplitude = 1.5709 ± 0.0632 V, $n = 8$) at urotensin II concentration of 10 nM, as compared to 0.3139 ± 0.0106 V for baseline (no human urotensin II added, $n = 8$, Fig. 3A). In the absence of human urotensin II, no phasic oscillations were observed. This type of urotensin II-induced oscillation could be completely inhibited by the calcium channel blockers: verapamil (10^{-6} M), nitrendipine (10^{-5} M), and diltiazem (10^{-5} M) (Fig. 3; Table 2). When tested at concentrations higher than 1 μM, all these inhibitors generated significant decreases of basal aorta ring tension (relaxation). The protein kinase C inhibitor, chelerythrine (2.5×10^{-5} M) and NPC-15437 (10^{-5} M) completely inhibited human urotensin II-induced phasic oscillations in high potassium Krebs buffer (Table 2; Fig. 3D and E). In contrast, the presence of tyrosine kinase inhibitors, tyrphostin B42 (1×10^{-6}–1×10^{-4} M) and genistein (1×10^{-6}–5×10^{-5} M) had no effect on amplitude or frequency of human urotensin II-induced phasic oscillations of the aorta rings (Table 2; Fig. 3F). Pre-incubation of the aorta rings in high potassium Krebs buffer in the presence of U-73122 (5×10^{-6} M) for 15 min or addition of the phospholipase C inhibitor after the phasic oscillations had developed, resulted in a complete blockade of human urotensin II-induced phasic oscillations (Table 2; Fig. 3G). HA 1077, a potent inhibitor of RhoA-associated kinase activity, at the concentration range of 1×10^{-6}–2.5×10^{-5} M had no effect on human urotensin II-induced phasic aorta ring oscillations (Table 2; Fig. 3H). However, genistein and HA 1077, compounds which were inactive in the inhibition of human urotensin II-induced phasic aorta ring oscillations in the high potassium Krebs buffer, significantly decreased basal aorta ring tension (Fig. 3F and H).

Binding affinities of the selected somatostatin-receptor antagonists to cloned rat and human urotensin II receptors displayed significant variations (Table 3). The only linear somatostatin analogue PRL-2814 displayed very weak affinities to all five somatostatin receptors as well as to cloned rat and human urotensin II receptors. Somatostatin receptor antagonists: PRL-2851, PRL-2970, PRL-3155 and PRL-3195 displayed weak affinities to both rat and human urotensin II receptors with some species preferences. PRL-3195, a somatostatin receptor antagonist displaying high affinities to the cloned human sst_5, sst_2 and sst_3 receptors, demonstrated much higher affinity to cloned rat urotensin II receptor as compared to cloned human urotensin II receptor (Table 3), while PRL-2970 seemed to have higher affinity for human urotensin II receptor (Table 3). Comparison of the binding affinities of the selected group of human somatostatin receptor antagonists for cloned rat and human urotensin II receptors allowed us, however, to identify the group of somatostatin receptor antagonists (PRL-2903, PRL-2915, PRL-3195) with relatively strong affinities to both rat and human cloned urotensin II receptors. Somatostatin antagonists PRL-2903 and PRL-2915 are sst_2 receptor preferring with low affinities for sst_3 and sst_5 (Table 3). Introduction of the somatostatin-receptor antagonist analogues at the concentrations range of 0.3 nM–30 mM into the regular incubation medium, after human urotensin II-induced aorta ring contractions had reached plateau, was without effect. However, pre-incubation of the aorta rings in the presence of PRL-2882, PRL-2903, and PRL-2915 (15 min at concentrations of 0.3 nM–30 mM) dose-dependently blocked the development of human urotensin II-induced tonic contractions by 90.6 ± 0.6, 82.3 ± 7.3; and 73.8 ± 7.7% (Fig. 4). In the high potassium Krebs buffer, addition of the selective somatostatin receptor antagonists at nanomolar or/and micromolar doses after human urotensin II-induced phasic oscillations had developed, resulted in a dose-dependent attenuation or complete inhibition of responses (Table 3; Fig. 5).

4. Discussion

Human urotensin II is a potent vasoactive peptide whose biological activity depends on the intact core octapeptide sequence, Asp-c[Cys-Phe-Trp-Lys-Tyr-Cys]-Val-OH (or NH_2). Also previously, the goby urotensin II fragment 5–12 was the smallest peptide sequence required to induce full contractile responses in the rat thoracic aorta (Itoh et al., 1987). Our data show that human urotensin II has high affinity for both recombinant rat and human urotensin II receptor ($K_i = 2.41$ and 1.6 nM, respectively) and that both goby and human urotensin II bind with much the same affinities to the rat recombinant urotensin II receptor. Elimination of the N-terminal Glu-Thr-Pro-tripeptide to create human urotensin II(4–11)-OH increased affinity to the rat recombinant UII receptor and also increased vasoconstrictor activity on the rat thoracic aorta. This observation might suggest that the elimination of the species-specific N-terminal sequence generates a more "neutral" across species urotensin II molecule with fully preserved vasoconstrictor activity.

It is important to note, however, that the vasoconstrictor activity of human urotensin II displays significant anatomical and species specific variations (Douglas et al., 2000) and that the peptide might actually induce vasodilator

responses in human small pulmonary and abdominal resistance arteries (Stirrat et al., 2001) and pre-contracted rat mesenteric and basilar arteries (Bottrill et al., 2000). Urotensin II-induced tonic rat aorta ring contractions could be partially and dose-dependently inhibited by Ca^{2+} channel inhibitors, protein kinase C inhibitors, and the specific tyrosine kinase inhibitors, tyrphostin B42 and genistein. Previous observations that tyrosine kinase inhibitors markedly inhibited agonist-induced constrictions in vascular smooth muscles were made using canine carotic arterial smooth muscles (DiSalvo et al., 1993) and rat aorta (Abebe and Agrawal, 1995). Recently, it was reported that genistein and tyrphostin A-23 diminished the contractile responses induced by $CaCl_2$ in the presence of norepinephrine in human small omental arteries and norepinephrine and the thromboxane A_2 analog U-46619 induced increases in the tyrosine phosphorylation of 42 and 58 kDa proteins, respectively, which could be prevented by genistein and tyrphostin A-23 (Martinez et al., 2000). These results suggest that tyrosine kinases might be implicated in the agonist-induced Ca^{2+} sensitization in arterial smooth muscle contractions. However, until now no experimental evidence has been presented that tyrosine kinase inhibitors are able to completely reverse human urotensin II-induced rat aorta ring contraction. Human urotensin II-induced tonic aorta ring contractions could be completely reversed by D609, a phospholipase C inhibitor that selectively inhibits phosphatidylcholine-specific phospholipase C, and was partially prevented by pre-incubation with ET-18-OCH_3, a phospholipase C inhibitor that selectively inhibits phosphatidylinositol-specific phospholipase C. These results support a previous report by Opgaard et al. (2000) indicating that human urotensin II directly increased labeled inositol phoshates in rabbit aortic slices and that both the phosphoinositide and constrictor responses appear to be mediated through phospholipase C. HA-1077 and a pyridine derivative, Y-27632, both potent RhoA-kinase (ROK) inhibitors, are known to potently inhibit smooth muscle contraction by inhibiting Ca^{2+} sensitization in the vascular system. Human urotensin II-induced tonic rat aortal ring contractions were completely inhibited by the Rho-associated kinase inhibitor H-1077. Thus, these results indicate that a ROK-associated pathway is involved in human urotensin II-induced rat aorta ring contractions. Recently, Sauzeau et al. (2001) demonstrated that human urotensin II induced arterial smooth muscle contraction as well as actin cytoskeleton organization and that arterial smooth muscle cell proliferation was mediated through the activation of RhoA and its effector, Rho-kinase. The RhoA/Rho-kinase-dependent contracting effect of human urotensin II is related to the phosphorylation and inhibition of myosin light chain phosphatase activity thus causing an increased myosin light chain phosphorylation and tension (Sauzeau et al., 2001). HA-1077 and Y-27632, the ROK-associated pathway inhibitors, were recently successfully applied to studies on human small omental arteries (Martinez et al., 2000) and in basilar artery studies (Chrissobolis and Sobey, 2001) which provided more supporting evidence that RhoA–Rho-kinase system is a major regulator of vascular smooth muscle contraction. The potential regulatory role of human urotensin II in these arteries is not known yet.

In high potassium Krebs buffer, which causes depolarization of the smooth muscle plasma membrane, human urotensin II generated phasic oscillations similarly to cholecystokinin, arginine vasopressin and bombesin (Yule and Williams, 1992; Schofl et al., 2000). Calcium channel blockers, phospholipase C inhibitors and protein kinase C inhibitors dose-dependently attenuated and inhibited human urotensin II-induced phasic aorta ring oscillations. These results imply that the phosphoinositide signaling pathway is activated by human urotensin II in aorta smooth muscle cells in high potassium Krebs buffer. In contrast, tyrosine kinase inhibitors did not inhibit human urotensin II-induced phasic oscillations, which in turn suggests that, under these conditions, the tyrosine kinase pathway is not activated. These observations are supported by the results of Abebe and Agrawal (1995) in which genistein and tyrphostin partially inhibited norepinephrine-stimulated rat aorta contractions but had no significant effects on contractile responses induced by potassium and phorbol 12,13-dibutyrate. Also, in human small omental arteries the tyrosine kinase inhibitors did not significantly modify the responses induced by norepinephrine and thromboxane A2 analogue U-46619 in high potassium medium and no increase in protein tyrosine phosphorylation was found (Martinez et al., 2000). In the high potassium medium, the RhoA-kinase inhibitor HA-1077 had no effect on human urotensin II-induced phasic oscillations but decreased basal tension of aorta rings. All these results suggests that human urotensin II-induced phasic oscillations of rat aorta rings are calcium channel and protein kinase C-dependent and are not mediated through tyrosine kinase and RhoA-kinase pathways.

Our studies indicate that several somatostatin antagonist analogues, highly structurally related to the urotensin II core sequence, were able to displace radioiodinated urotensin II from human and rat recombinant urotensin II receptor binding sites and that the displacement potency correlate reasonably well with the ability of somatostatin analogues to inhibit human urotensin II-induced aorta ring tonic contractions. Somatostatin analogues PRL-2882, PRL-2903 and PRL-2915 were able to block human urotensin II-induced rat aorta ring tonic contractions, (EC_{50} values are 3.1, 3.3 and 8.7×10^{-6} M, respectively) and appear to be the first reported urotensin II receptor antagonists.

In addition, PRL-2903 was found to be effective in the inhibition of [^{125}I]goby-urotensin II binding to rat heart membranes (data not shown). Possible interaction of somatostatin and somatostatin analogues with urotensin II receptors was previously reported by Loretz (1990) who found that a somatostatin octapeptide analogue, SMS 201–995, mimicked the effect of urotensin II by increasing urotensin II-dependent Cl^- absorption in goby intestine. These results

are also consistent with a previous report by Liu et al. (1999) that urotensin II receptors could be activated by somatostatin analogues at high, micro-molar concentrations. In contrast to previously used somatostatin peptides (Traina and Bagnoli, 1999; Loretz, 1990; Liu et al., 1999), all presently studied analogues were demonstrated to be somatostatin receptor antagonists also, with the highest affinities to $hsst_2$ and $hsst_5$ subtypes (Hocart et al., 1998, 1999). We have found also that several somatostatin analogues were able to inhibit human urotensin II-induced phasic oscillations in high K^+ Krebs buffer (Table 3; Fig. 5). These human urotensin II-induced phasic oscillations, which we have shown are calcium L-channel and protein kinase C-dependent, could also be inhibited by sst_2 antagonists perhaps through the mechanisms similar to those observed in pituitary tumor cells (Petrucci et al., 2000). It was previously shown that somatostatin and SRIF-analogues related to octreotide (SMS 201–995), and BIM-23056, at micro-molar concentrations in high potassium medium, significantly reduced $[Ca^{2+}]_i$ in PC12 cells (Traina and Bagnoli, 1999) and that somatostatin-induced inhibition of Ca^{2+} influx in pituitary tumor cells was mediated by activating sst_2 receptors and voltage-dependent L-type Ca^{2+} channels (Petrucci et al., 2000). In isolated rat thoracic aorta smooth muscle cells, somatostatin induced dose-dependent reduction of the cell-surface area, elevation of cytosolic Ca^{2+} and stimulation of myosin light chain (MLC) phosphorylation (Torrecillas et al., 1999). It was recently reported that somatostatin stimulates arachidonic acid mobilization and that sst_2 and sst_5 are involved in this process (Alderton et al., 2001). In addition, in the presence of vasoconstrictors involving activation of protein kinase C, octreotide exerts a local vasoconstrictive effect on vascular smooth muscle of the superior mesenteric artery (Wiest et al., 2001). We suggest, therefore, that somatostatin analogues with preferential affinity for sst_2 and sst_5 receptor subtypes might indeed be the most likely to display effects on vascular smooth muscle.

We conclude that: (1) Human urotensin II and its N-terminally deleted human urotensin II-(4–11)-OH/NH_2 analogues are potent vasoconstrictor peptides in the rat thoracic aorta ring contraction model. (2) Human urotensin II-induced tonic aortal smooth muscle contraction is mediated through the activation of Ca^{2+} channels, RhoA-kinase, protein kinase C, tyrosine kinase and phospholipase C pathways. (3) In the presence of high K^+, human urotensin II induced concentration-dependent aortal smooth muscle phasic oscillations which require active Ca^{2+} channels and activation of phospholipase C and protein kinase C pathways. (4) Human urotensin II binding to the cloned rat and human urotensin II receptors could be displaced by somatostatin antagonist analogues preferentially recognizing sst_2. (5) Human urotensin II binding to the rat aortal smooth muscle cells could be antagonized (blocked) by the somatostatin antagonist analogues preferentially recognizing sst_2. We are hopeful that the present weak affinity somatostatin-based urotensin II antagonists can form a structural template for the development of more potent urotensin II receptor antagonists of therapeutic interest in the treatment of cardiovascular disease.

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