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Human urotensin II mediates vasoconstriction via an increase in inositol phosphates

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Abstract

The cyclic peptide urotensin II has recently been cloned from human and reported to potently constrict primate blood vessels. To elucidate the cellular signalling mechanisms of this peptide, we investigated a possible relationship of vasomotor effects of human urotensin II and phosphoinositide turnover in isolated rabbit thoracic aorta. Human urotensin II produced a slowly developing increase in isometric contractile force ($pEC_{50} = 9.0$) that was endothelium-independent. The contractile effect of urotensin II was significantly inhibited by the phospholipase C inhibitor, 2-nitro-4-carboxyphenyl-*N*,*N*,-diphenylcarbamate (NCDC), but not by the cyclooxygenase inhibitor, indomethacin. In slices of rabbit thoracic aorta, human urotensin II increased phosphoinositide hydrolysis, and this effect was also inhibited by NCDC. The potency of urotensin II ($pEC_{50} = 8.6$) was similar to that found in the contractile studies. Thus, vasoconstrictor effects of human urotensin II appear to be mediated by a phospholipase C-dependent increase in inositol phosphates, suggesting that the peptide acts via a G_q protein-coupled receptor.

Keywords: Rabbit; Artery; Phosphoinositide; Peptide; Phospholipase C; Urotensin II

1. Introduction

Urotensin II was recently discovered in mammals, including humans (Coulouarn et al., 1998); and its receptor has now been identified as the orphan receptor GPR14 (Ames et al., 1999; Liu et al., 1999; Mori et al., 1999; Nothacker et al., 1999). These findings have renewed interest in urotensin II, long thought to be a neuropeptide restricted to lower vertebrates. Urotensin II was originally detected in teleost fish, where it was isolated from the urophysis as a cyclic, 12-amino acid peptide (Berlind, 1972; McMaster and Lederis, 1983). More recently, urotensin II has been characterized in frog (Conlon et al., 1997), rat, mouse (Coulouarn et al., 1999) and pig (Mori et al., 1999). The cDNA encoding the precursor to human urotensin II, preprourotensin, has now been reported along with the localization of its corresponding mRNA in human spinal cord and kidney (Coulouarn et al., 1998). Human urotensin II has only 11 amino acids; but the key cyclic region of the peptide responsible for biological activity has been conserved from fish to human (Coulouarn et al., 1998).

Earlier work with fish and frog urotensin II demonstrated cardiovascular effects of this peptide (Gibson, 1987; Gibson et al., 1988, 1986; Hazon et al., 1993; Itoh et al., 1987, 1988; Le Mevel et al., 1996; Yano et al., 1995). We synthesized human urotensin II as proposed from the structure of the human urotensin II precursor and found it to be a potent constrictor of isolated rat thoracic aorta (Nothacker et al., 1999). Recently published studies described contractile effects of human urotensin II on various isolated arteries from monkeys (Ames et al., 1999) and the rat main pulmonary artery (MacLean et al., 2000). Furthermore, systemic administration of human urotensin II to monkeys elicited a complex dose-dependent haemodynamic response that culminated in severe myocardial depression and fatal circulatory collapse (Ames et al., 1999). These striking cardiovascular effects suggest urotensin II may have important clinical significance.

The cellular signalling mechanisms of urotensin II have not been established, although the vasoconstrictor effects have been proposed to involve calcium (Gibson et al., 1988; Itoh et al., 1987) and possibly arachidonic acid

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metabolites (Yano et al., 1995). The urotensin II receptor GPR14 appears to be coupled through $G_{q/11}$ proteins to elevate intracellular calcium in cultured cells (Liu et al., 1999; Nothacker et al., 1999). We recently detected mRNA for GPR14 in rat aorta (Nothacker et al., 1999). The aim of the present study was to investigate possible second messenger pathways mediating vasoconstrictor effects of human urotensin II. We hypothesized that urotensin II activates phospholipase C leading to an increase in inositol phosphates and vascular tone. To test this, we measured both contractile and phosphoinositide responses of human urotensin II in isolated rabbit arteries, and assessed the effects of the cyclooxygenase inhibitor indomethacin and the phospholipase C inhibitor 2-nitro-4-carboxyphenyl-*N,N*,-diphenylcarbamate (NCDC) (Bakker et al., 1999; Walenga et al., 1980).

2. Materials and methods

Male rabbits were euthanized with CO_2, and the thoracic aorta, pulmonary artery, ear artery and proximal coronary arteries were rapidly dissected out and placed in Krebs buffer of the following composition (mM): NaCl 118, KCl 5.2, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25.5, $CaCl_2$ 1.6, disodium EDTA 0.027, glucose 1.2.

2.1. Measurement of contractile force

Vessel segments with a length of 1–2 mm were excised and mounted in temperature-controlled tissue baths (37°C), where each segment was connected to a transducer for recording of isometric circular wall tension. The tissue bath contained Krebs buffer that was continuously bubbled with a mixture of 95% O_2/5% CO_2, giving a pH of approximately 7.4. Each vessel segment was stretched to a designated resting tension, conveniently described here in mass equivalents: coronary artery, 0.8 g; ear artery, 1 g; pulmonary artery, 1.5 g; and thoracic aorta, 2 g. Due to initial spontaneous relaxations of the vessels, it was necessary to make several adjustments of the applied force in order to maintain a stable resting tension. After 1 1/2 h, when the tension had stabilized at the desired level, the vessel was exposed to a modified Krebs buffer containing 90 mM KCl, obtained by substituting equimolar concentrations of NaCl with KCl. Only those vessels that responded to 90 mM KCl with a contraction were used for further investigation. The tension induced in each individual segment by the potassium-rich solution was set arbitrarily to 100% and used as an internal standard, to which the agonist-induced contractions were compared. The endothelium of some artery segments was removed by rubbing the lumen of the artery with a wire. The absence of endothelium in these artery segments was confirmed by the loss of dilator responses to acetylcholine (10^{-5} M) after precontraction with 90 mM K^+ or 10^{-6} M phenylephrine. When testing the effects of indomethacin or NCDC, the inhibitor was added to the tissue bath 20 min prior to addition of human urotensin II. The concentration of NCDC (10^{-4} M) was selected to produce near-maximal inhibition of phospholipase C-mediated vasoconstriction (Bakker et al., 1999; Florian and Watts, 1998; Kim et al., 2000).

2.2. Phosphoinositide hydrolysis

The procedures for measuring phosphoinositide hydrolysis were adapted from methods previously described (Batty and Nahorski, 1985; Berridge et al., 1982; Garcia-Villalon et al., 1990). Segments of the thoracic aorta were cut open and placed on a McIlwain tissue chopper (Brinkmann Instruments, Westbury, NY). The segments were chopped twice at a setting of 350 μm, with the stage being rotated 90° between each run. The slices were placed immediately in Krebs buffer that was gassed with 95% O_2/5% CO_2 and kept at a temperature of 37°C. The tissue was washed three times with fresh, warm Krebs buffer, and the slices were allowed to settle between each wash. The slices were then incubated in gassed, warm Krebs buffer for 30 min. The slices were washed again in fresh Krebs buffer and resuspended in 10 ml of Krebs buffer containing 100 μl [^{3}H]inositol (i.e., 100 μCi), followed by incubation in this solution, which was gassed and kept at 37°C, for 3 h. Following the labelling period, the tissue was washed twice in warm, gassed Krebs buffer, and a third washing was performed using Krebs buffer containing 10 mM LiCl. The supernatant was removed, and aliquots (35 μl each) of gently packed tissue slices were added to tubes containing Krebs buffer with 10 mM LiCl and drugs as needed in a final volume of 0.35 ml. The samples were incubated for 1 h while gassed and kept at 37°C. The incubation was stopped by adding 1.1 ml chloroform/methanol (1:2, v/v), and the slices were allowed to stand for 15 min. To separate aqueous and organic phases, 0.37 ml of water and 0.37 ml of chloroform were added to each tube; and the tubes were capped, shaken and then centrifuged at $2000 \times g$ for 2 min. An aliquot (1 ml) of the aqueous phase (upper phase) was removed and placed in a tube with an additional 2 ml of water, whereas 200 μl of the organic phase (lower phase) was placed into a scintillation vial, and the chloroform was allowed to evaporate. The tubes containing the aqueous phase were centrifuged at $2000 \times g$ for 2 min. Nearly all (2.8 ml) of the aqueous phase was collected from each tube and applied to a column of 1 ml of Dowex AG 1-X8 (formate form, 100–200 mesh). The columns were washed four times with 4 ml of water each time. The columns were then placed on top of scintillation vials, and [^{3}H]inositol phosphates were eluted from the column with 2.5 ml of a mixture of 1 M ammonium formate and 0.1 M formic acid. An aliquot (10 ml) of scintillation cocktail was added to each vial, and the radioactivity was measured for determination of [^{3}H]inositol phosphates. Scintillation cocktail

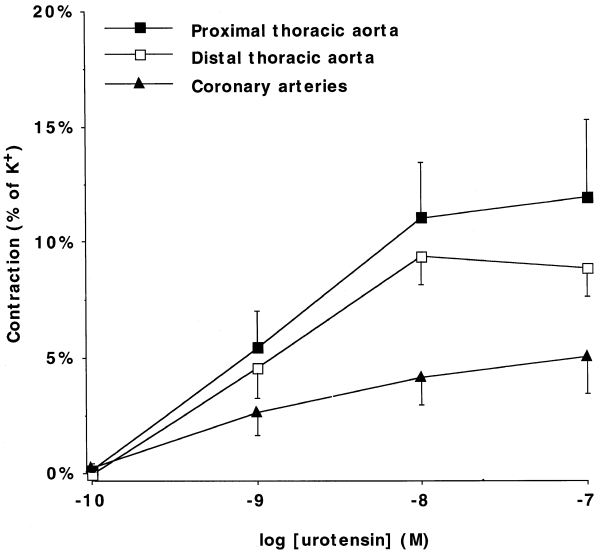

Fig. 1. Contractile effect of human urotensin II on isolated proximal (aortic arch) and distal descending portions of the rabbit thoracic aorta as well as segments of rabbit coronary arteries. A contractile effect was seen in all aortic segments ($n = 11$ segments from five rabbits in each group), and in 10 coronary segments out of 18 tested from six rabbits. The contraction to urotensin II is expressed as a percentage of the K^+-induced (90 mM) contraction in the same vessel segment. The curves show mean ± S.E.M. of all vessel segments responding to human urotensin II.

(4 ml) was added to each tube containing the organic phase after the chloroform had evaporated, and the radioactivity was measured for determination of labeled phosphoinositides. The [^{3}H]inositol phosphates are expressed relative to the total amount of [^{3}H]phosphoinositides plus [^{3}H]inositol phosphates to correct for variation in the amount of tissue added to each tube (Ehlert et al., 1996).

2.3. Analysis of data

Maximum contractile effect obtained with an agonist, E_{max}, and the negative logarithm of the concentration of agonist that elicited half-maximum effect, pEC_{50}, were derived from concentration–response curves on each vessel segment. Values are given as mean ± S.E.M. One factor analysis of variance (ANOVA) followed by Fisher's Protected Least Significant Difference test was used to determine the statistical significance with respect to differences in E_{max} and pEC_{50} values. Statistical significance was assumed when $P < 0.05$.

2.4. Drugs

Sigma Chemical Co. (St. Louis, MO): sodium carbonate (Na_2CO_3), L-phenylephrine HCl, indomethacin, NCDC, acetylcholine HCl. Human urotensin-II was chemically synthesized as previously described (Nothacker et al., 1999). Fisher Scientific (Pittsburg, PA): lithium chloride (LiCl) and salts for Krebs solution. New England Nuclear (Boston, MA): *myo*-[2-^{3}H(N)]-inositol (21 Ci/mM, 1 mCi/ml). Indomethacin was dissolved in 0.1 M sodium carbonate and NCDC was dissolved in ethanol prior to dilution in Krebs buffer. All the other drugs were dissolved in water.

3. Results

3.1. Vasomotor effects

All isolated segments of rabbit thoracic aorta contracted to cumulative concentrations of human urotensin II (10^{-10} to 10^{-7} M). The increase in tension was very slow, taking approximately 20 min to develop for each concentration of human urotensin II. Contractions were also slow to reverse following washout of the peptide. Segments of the proximal (aortic arch) and distal (descending aorta above diaphragm) thoracic aorta gave similar responses to urotensin II (Fig. 1). Proximal coronary arteries also responded to human urotensin II with a slowly developing contraction (10 out of a total of 18 artery segments tested from six rabbits, Fig. 1). The potency of urotensin II was similar in the coronary artery and thoracic aorta, but the maximal response was smaller in the coronary artery segments (Table 1).

No significant contractile responses to urotensin II (10^{-12} to 10^{-7} M) were detected in either the pulmonary artery (10 segments from four rabbits) or the ear artery (eight segments from three rabbits). In order to detect possible relaxant effects, human urotensin II (10^{-12} to 10^{-7} M) was also tested on ear artery segments after precontraction with phenylephrine (10^{-6} M), but no relaxant responses were seen (eight segments from four rabbits). Finally, human urotensin II (10^{-12} to 10^{-7} M) was tested on three segments of rabbit ear vein, but no change in vasomotor tone was observed. Because it gave the best response to urotensin II, all subsequent experiments were performed using segments of the thoracic aorta.

In order to assess whether urotensin II acted directly on the smooth muscle, we compared endothelium-intact and endothelium-denuded segments from the middle part of the descending thoracic aorta (Fig. 2). Although there was a tendency for maximal responses to be greater in denuded vessels (21.6 ± 5.9 vs. 13.6 ± 3.6, $n = 9$ segments from

Table 1
Contractile effects of human urotensin II in the rabbit thoracic aorta and coronary arteries

Vessel location	Rabbits	Vessel segments	K^+ (g)	Human urotensin II	
				E_{max} (%)[a]	pEC_{50}
Proximal aorta	5	11	3.7 ± 0.4	12.7 ± 3.3	9.09 ± 0.16
Distal aorta	5	11	4.1 ± 0.3	9.4 ± 1.2	8.94 ± 0.12
Coronary arteries	6	10	1.7 ± 0.2	5.5 ± 1.5[b]	9.10 ± 0.21

The values are given as mean ± S.E.M. of all vessel segments tested.
[a] % of K^+-induced contraction.
[b] $P < 0.05$ compared to aortic segments.

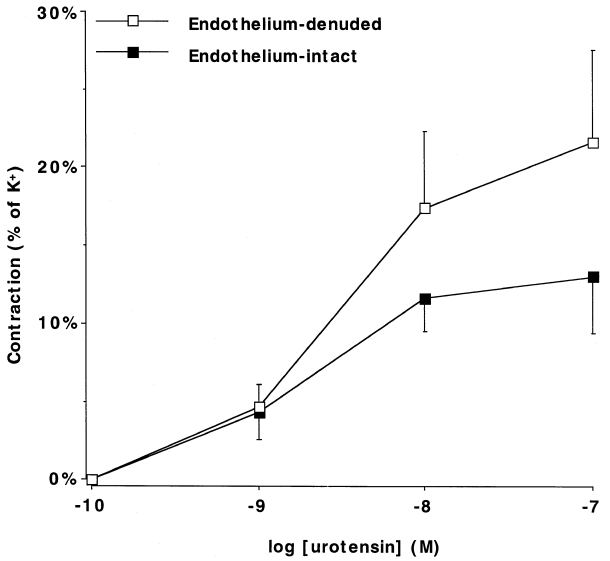

Fig. 2. Contractile effect of human urotensin II on endothelium-intact vs. endothelium-denuded segments of the descending rabbit thoracic aorta. Contraction to urotensin II is presented as the percentage of K^+-induced (90 mM) contraction in the same vessel segment (K^+-induced contraction 4.0 ± 0.05 g in endothelium-intact arteries and 4.6 ± 0.9 g in endothelium-denuded arteries). The curves represent mean ± S.E.M. of all vessel segments ($n = 9$ segments from three rabbits in each group).

three rabbits), there were no significant differences between the effects of urotensin II on intact and endothelial-denuded aorta segments (pEC_{50}: 8.80 ± 0.14, intact; 8.66 ± 0.13, denuded).

Table 2
Effect of NCDC and indomethacin on the contractile effects of human urotensin II in rabbit thoracic aorta

Treatment	Rabbits	Vessel segments	K^+ (g)	Human urotensin II E_{max} (%)
Control	4	8	4.2 ± 0.5	13.7 ± 3.1
NCDC	4	8	4.1 ± 0.6	2.4 ± 2.0[a]
Indomethacin	4	8	4.3 ± 0.7	11.6 ± 5.0

The values are given as mean ± S.E.M. of all vessel segments tested. All segments responded with a contraction to human urotensin II, except in the group pre-incubated with NCDC, where only four out of eight segments responded to human urotensin II.
[a] $P < 0.05$ compared to control.

The phospholipase C inhibitor NCDC (10^{-4} M) significantly inhibited contractile responses to human urotensin II (Fig. 3), actually abolishing the response in four of the eight aorta segments tested. The E_{max} value for human urotensin II was significantly decreased after pre-incubation with NCDC (Table 2), but there was no significant change in pEC_{50} values: 8.30 ± 0.29 with NCDC vs. 8.93 ± 0.20 in the controls. In contrast, pre-incubation with the cyclooxygenase inhibitor indomethacin (10^{-5} M) did not significantly affect contractile responses to human urotensin II. All segments responded to human urotensin II, and the E_{max} values were similar with or without indomethacin (Table 2). The pEC_{50} also did not change significantly: 8.70 ± 0.08 with indomethacin vs. 8.93 ± 0.20 in the controls.

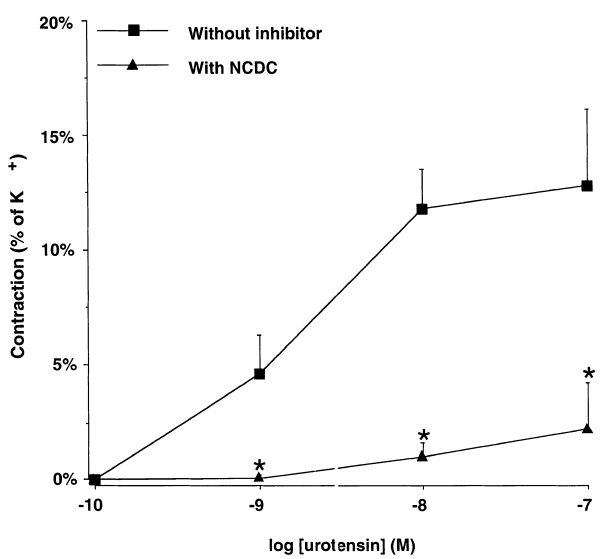

Fig. 3. Effect of the phospholipase C inhibitor NCDC (10^{-4} M) on contractile responses to human urotensin II in segments of rabbit descending thoracic aorta. $n = 8$ vessel segments from four rabbits in each group. A response to human urotensin II was seen in all segments without the inhibitor but in only four out of eight vessel segments pre-incubated with NCDC. Contraction to urotensin II is presented as percentage of K^+-induced (90 mM) contraction in the same vessel segment, and the curves represent the mean ± S.E.M. of all vessel segments tested. * Significantly different from segments without inhibitor ($P < 0.05$).

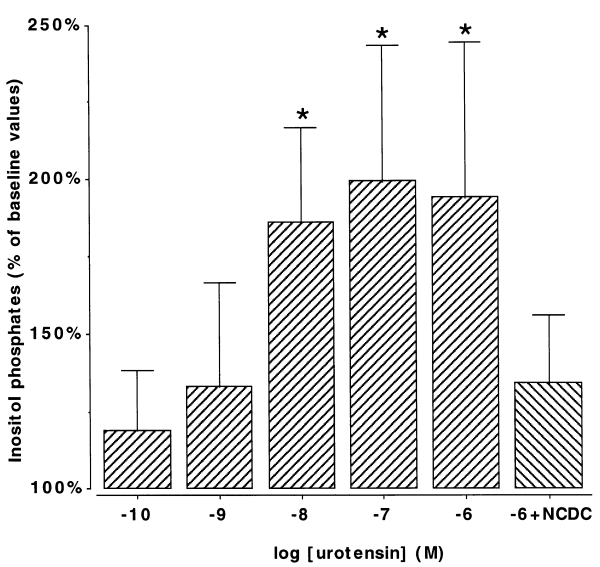

Fig. 4. Effect of increasing concentrations of human urotensin II on phosphoinositide hydrolysis in the rabbit thoracic aorta. Levels of [^{3}H]inositol phosphates are expressed relative to the total amount of [^{3}H]phosphoinositides plus [^{3}H]inositol phosphates and presented as percentage of baseline values obtained in the absence of human urotensin II. Each bar represents the mean ± S.E.M. of four experiments (four rabbits), each experiment done in triplicate. * Significantly different from baseline values ($P < 0.05$).

3.2. Phosphoinositide hydrolysis

In slices of rabbit thoracic aorta, human urotensin II (10^{-10} to 10^{-6} M) increased the level of [^{3}H]inositol phosphates in a concentration-dependent manner (Fig. 4). The maximum effect ($199 \pm 4\%$ of baseline value) was observed at a concentration of 10^{-7} M. The pEC_{50} value for human urotensin II was 8.61 ± 0.17. However, in the presence of NCDC (10^{-4} M), human urotensin II (10^{-6} M) had no significant effect on inositol phosphates.

4. Discussion

In the present study of rabbit thoracic aorta, we demonstrate that human urotensin II increases phosphoinositide turnover with the same potency that it causes vasoconstriction. Both effects are inhibited by the phospholipase C inhibitor NCDC, further indicating a role of inositol phosphates in the vascular effects of urotensin II (Bakker et al., 1999; Turla and Webb, 1990). This study is the first work to establish the phosphoinositide signal transduction pathway for urotensin II, and it is consistent with an action of urotensin II on $G_{q/11}$-coupled receptors such as GPR14 (Liu et al., 1999; Nothacker et al., 1999).

Nanomolar concentrations of human urotensin II produced a sustained increase in vascular tone in isolated rabbit thoracic aorta and coronary arteries. The potency of human urotensin II and the slowly developing nature of the contraction are analogous to what was observed recently in rat thoracic aorta (Nothacker et al., 1999). Human urotensin II also has been shown to potently constrict various arteries from non-human primates (Ames et al., 1999). Similar vascular effects were found for frog and fish urotensin II when these peptides were tested on frog and fish arteries (Le Mevel et al., 1996; Yano et al., 1995) and when fish urotensin II was used to constrict rat (Itoh et al., 1987) and rabbit (Muramatsu et al., 1979) isolated aorta. Vasorelaxant effects of low concentrations (0.1–0.5 nM) of fish urotensin II on isolated rat aortic strips also have been reported (Gibson, 1987); however, we did not detect relaxation to human urotensin II in the present study.

Interestingly, contractile effects of urotensin II appear to vary among vascular beds. As in our rabbit study, the strongest contractions to urotensin II have generally been measured using thoracic aorta (Itoh et al., 1987, 1988). In our study, a contractile effect of urotensin II was also observed on rabbit coronary arteries, although the degree of constriction of the isolated vessels was small. In contrast, neither rabbit pulmonary artery, ear artery, nor ear vein responded to human urotensin II. Other studies have noted the lack of venous responses to urotensin II (Le Mevel et al., 1996). Recently, MacLean et al. (2000) reported human urotensin II constricts the rat main pulmonary artery but has no effect on smaller pulmonary arteries. The varying levels of constriction may reflect differences in the expression of vascular urotensin II receptors. Itoh et al. (1988) found the number of binding sites for radiolabelled fish urotensin II varied among rat arteries and correlated with the degree of constrictor response (thoracic aorta > abdominal aorta > mesenteric artery). Given that the cyclic structure of the peptide is highly conserved (Coulouarn et al., 1998), it is likely that fish and human urotensin II act via the same receptor in mammalian arteries. Because urotensin II produced contraction in the absence of the endothelium, it appears that urotensin II receptors are located on vascular smooth muscle.

The orphan G-protein-coupled receptor GPR14, which was cloned from rat (Marchese et al., 1995; Tal et al., 1995) and human (Ames et al., 1999), has been identified as a putative receptor for urotensin II (Ames et al., 1999; Liu et al., 1999; Mori et al., 1999; Nothacker et al., 1999). The mRNA for GPR14 is expressed in cardiovascular tissue, suggesting this receptor is present in various arteries as well as the heart (Ames et al., 1999; Liu et al., 1999; Nothacker et al., 1999; Tal et al., 1995). We previously found GPR14 mRNA in the thoracic aorta of rat (Nothacker et al., 1999). GPR14 mRNA also has been detected in human arterial tissue, but not in the vena cava or in a venous (renal vein) smooth muscle cell line (Ames et al., 1999).

The present study is to our knowledge the first to demonstrate the involvement of phosphoinositide hydrolysis as a second messenger pathway mediating contractile responses to urotensin II. This conclusion is based on our finding that human urotensin II directly increased labelled inositol phosphates in rabbit aortic slices treated with LiCl (Berridge et al., 1982). The effect was concentration-dependent with a pEC_{50} value (8.6) for urotensin II that correlated with the potency of this peptide for constricting the aorta (pEC_{50} of 9.0). Furthermore, both the phosphoinositide and constrictor responses to urotensin II appear to be mediated through phospholipase C because they were inhibited by NCDC (Bakker et al., 1999; Turla and Webb, 1990; Walenga et al., 1980). Signal transduction via phospholipase C suggests that urotensin II may act via a G_q protein-coupled receptor (Hamm, 1998; LeVine, 1999).

Activation of phospholipase C leads to elevation of intracellular calcium, which causes contraction in vascular smooth muscle. Thus, our findings are consistent with previous studies demonstrating that urotensin II increases ^{45}Ca influx in rat aorta (Gibson et al., 1988) and elevates intracellular calcium in cultured cells transfected with GPR14 (Ames et al., 1999; Liu et al., 1999; Nothacker et al., 1999). The responses of GPR14 in the latter assay are also consistent with coupling to G_q proteins (Hamm, 1998; LeVine, 1999). The high potency of human urotensin II on GPR14-transfected cells (pEC_{50} of 9.1–10) correlates with what was found for contractile and phosphoinositide responses to urotensin II in rabbit aorta. It has also been reported that urotensin II increases the release of arachidonic acid from Chinese hamster ovary cells transfected

with GPR14 (Mori et al., 1999); and in frog arteries, constriction to urotensin II was inhibited by the cyclooxygenase inhibitor indomethacin (Yano et al., 1995). However, we did not find any effect of indomethacin on constriction of rabbit aorta by human urotensin II, similar to what was reported for fish urotensin II (Gibson, 1987; Hazon et al., 1993; Itoh et al., 1987). Thus, it appears that the phosphoinositide pathway is the primary signal transduction mechanism leading to vasoconstriction by urotensin II.

The overall significance of urotensin II in cardiovascular regulation has yet to be determined. Systemic injection of urotensin II results in complex effects that likely reflect both cardiac and vascular actions of the peptide (Ames et al., 1999; Gibson et al., 1986; Hazon et al., 1993; Le Mevel et al., 1996; Yano et al., 1995). Immunohistochemical studies demonstrated the presence of urotensin II-like immunoreactivity in human and monkey vasculature, and diffuse immunostaining was observed in human cardiomyocytes and coronary atherosclerotic plaque (Ames et al., 1999). It was further demonstrated that mRNA for the human urotensin II precursor is expressed in human kidney, suggesting this organ may be the source of circulating urotensin II (Nothacker et al., 1999). Characterizing the signal transduction pathways mediating the effects of urotensin II is an important step in understanding the functions of urotensin II. Our results in the rabbit aorta suggest the contractile effect of human urotensin II is mediated by a phospholipase C-dependent increase in inositol phosphates.

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