Ligands and Signaling of the G-Protein-Coupled Receptor GPR14, Expressed in Human Kidney Cells

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Key Words

Guanylin • Uroguanylin • Urotensin II • GPR14 • HEK293 cells • CHO cells • Patch-clamp technique • Arachidonic acid • Phospholipase A₂

Abstract

Activation of the urotensin II (U-II) receptor, GPR14, leads to an increase in Ca²⁺, activation of phospholipase A₂ (PLA₂) and an increase in arachidonic acid. The signaling pathway for guanylin peptides in the kidney involves an unknown G-protein coupled receptor which activates PLA₂ and increases arachidonic acid as well. To test if guanylin peptides could be, as U-II, agonists for the GPR14 receptor in the kidney, we used HEK293 and CHO cells transfected with hGPR14 (HEK293+hGPR14, CHO+hGPR14, respectively). Effects of guanylin peptides and U-II were studied by slow-whole-cell patch-clamp analysis and microfluorimetric measurements of intracellular Ca²⁺. Guanylin peptides and U-II depolarized HEK293+hGPR14 significantly more than wild type cells. These effects were inhibited in the presence of Ba²⁺ or PLA₂ inhibition (AACOCF₃), suggesting that guanylin peptides and U-II increase

arachidonic acid and inhibit ROMK channels in these cells. However, only U-II was capable to increase the cellular Ca²⁺, suggesting different mechanism of GPR14 activation by guanylin peptides and U-II. This signaling pathway of U-II involves PKC, because U-II effects in HEK293+hGPR14 cells were inhibited by calphostin C. Guanylin peptides activate PLA₂ and inhibit ROMK channels in HEK293 cells transfected with the human GPR14 receptor. Since GPR14 is present in mouse and human CCD it is a candidate for the guanylate cyclase independent receptor for guanylin peptides.

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Introduction

Urotensin (U-II) is a hexapeptid with one disulfide bond [1]. U-II vasoactivity differs between species and vascular beds [2]. U-II is highly expressed in the human kidney [3, 4] and causes natriuresis and vasodilations in rat kidney [5]. U-II is a ligand for the G-protein coupled receptor (GPR14) [6]. An activation of GPR14 leads to activation of phospholipase C (PLC), an increase in

intracellular Ca^{2+} , and activation of phospholipase A_2 (PLA_2) with an increase in cellular arachidonic acid [7]. mRNA for human GPR14 is expressed in human heart, kidney, brain, pancreas, skeletal muscle, vascular smooth muscle, endothelial cells, spinal cord and endocrine tissue [3,7,8]. This wide distribution of GPR14 suggests that U-II may have an endocrine function or GPR14 serves as receptor for other hormones.

Possible additional ligands for the GPR14 receptor could also be guanylin and uroguanylin (guanylin peptides). The intestine secretes guanylin peptides [9] which causes natriuresis, kaliuresis, and diuresis in the kidney [10-12]. In the intestine guanylin peptides regulate water and electrolyte transport via guanylate cyclase C (GC-C). However, in GC-C deficient mice (GC-C $-/-$) guanylin peptides still cause natriuresis, kaliuresis, diuresis and increased urinary cGMP levels. This suggests the existence of at least one additional signaling pathway in the kidney for guanylin peptides which is GC-C independent [12]. In a human proximal tubule cell line (IHKE1), we recently demonstrated that UGN activates two signaling pathways: one involving GC-C leading to an increase in cellular cGMP; the other involves the activation of a pertussis toxin (PT)-sensitive G-protein [13]. Results from principal cells of mouse and human cortical collecting ducts (CCD) suggest the existence of another signaling pathway for guanylin peptides involving the activation of phospholipase A_2 (PLA_2) and arachidonic acid [14, 15].

Therefore we examined if guanylin peptides activate GPR14 and thereby mediate the effects observed in the kidney. This was tested in human embryonic kidney cells (HEK293) and chinese hamster ovary cells (CHO) as expression systems, both transfected with the human GPR14 receptor.

Materials and Methods

Cell culture

HEK293 cells transfected with human GPR14 (kindly provided by Sanofi-Aventis, Höchst, Germany) as shown previously [16] were cultured on glass cover slips in DMEM with 3.7 g/L NaHCO_3 , 2 mM L-glutamine, 10 ml/l Penicillin/Streptomycin (10000 U/10000 $\mu\text{g}/\text{ml}$, Biochrom), 12 ml/l Geneticin (50mg/ml, Gibco, (Karlsruhe, Germany) and 10% FCS in an atmosphere with 8% CO_2 at 37°C. The HEK293 WT cells were kept in the same media with the exception of geneticin. The cells were used 5-12 days after trypsinization (0.05% trypsin, 0.02% EDTA in Mg^{2+} - and Ca^{2+} -free phosphate buffer).

CHO-K1 cells transfected with human GPR14 [17] (kindly provided by Actelion Ltd., Allschwil/Basel, Switzerland) were

cultured on glass cover slips in Iscoves medium with 3.0 g/L NaHCO_3 , 15 mg/L phenolred supplemented with 200 mM L-glutamine, 5 ml/l Penicillin/Streptomycin (10000 U/10000 $\mu\text{g}/\text{ml}$, Biochrom), geneticin (50 mg/ml), 50 ml of 10% fetal calf serum in an atmosphere with 8% CO_2 at 37°C. The CHO WT cells were kept in the same media only without geneticin. The cells were used 4 days after trypsinization (0.25% trypsin, 0.1% EDTA in Mg^{2+} - and Ca^{2+} -free phosphate buffer).

Patch-clamp technique

Coverslips with HEK293 or CHO cell monolayers were mounted at the bottom of a perfusion chamber of an inverted microscope (Axiovert 10, Zeiss, Göttingen, Germany). Membrane voltages were measured with the slow-whole-cell patch-clamp technique [18]. A modified Ringer solution was used for experiments containing: 145 mM NaCl, 1.6 mM K_2HPO_4 , 0.4 mM KH_2PO_4 , 5 mM D-glucose, 1 mM MgCl_2 , 1.3 mM calcium gluconate (pH 7.4). All test substances were dissolved in this Ringer solution. The filling solution for patch-clamp pipettes contained: 95 mM potassium gluconate, 30 mM KCl, 4.8 mM Na_2HPO_4 , 1.2 mM NaH_2PO_4 , 5 mM D-glucose, 0.73 mM calcium gluconate, 1 mM EGTA, 1.03 mM MgCl_2 , 1 mM ATP (pH 7.2). To this solution 160 μM Nystatin were added to allow electrical access to the cytosol. The membrane potential was measured with a patch clamp amplifier (U. Fröbe, Physiologisches Institut, Universität Freiburg, Germany) and recorded continuously with a pen chart recorder (WeKa graph WK-250R, WKK, Kaltbrunn, Switzerland).

The involvement of K^+ conductances was evaluated by the use of two inhibitors of K^+ channels, Ba^{2+} (1mM) as general K^+ channel inhibitor and arachidonic acid (10 μM) as inhibitor of ROMK channels [19]. The involvement of PLA_2 and PKC in the regulation of the GPR14 receptor was determined using the specific inhibitors AACOCF₃ (5 μM) and calphostin C (100 nM), respectively. To determine whether the effects of U-II and UGN are dependent on intracellular Ca^{2+} stores thapsigargin (1 μM) was used. The involvement of the GPR14 receptor in the signaling pathway of U-II and UGN was further tested using BIM-23127 and urantide, both inhibitors of this receptor [20, 21].

RT-PCR

Nephron segments were isolated using the procedure described previously [22, 23]. In brief, cortical pieces of mouse and human kidney were incubated in modified Eagle's medium (MEM) containing 5 mM glycine, 480 IU/ml trypsin inhibitor from soybean, 50 IU/ml DNase, 178 IU/ml and 533 IU/ml collagenase type 2 (Worthington Biochemical Corp., New Jersey, USA) for mouse and human kidney, respectively, for 60 - 100 min at 37°C with aeration (95% O_2 and 5% CO_2). Specific nephron segments were collected in MEM with 5 mM glycine under 50 x magnification at 4°C.

Cortical pieces of human kidney, isolated nephron segments (approximately 40 mm), glomeruli (400 pieces) or HEK293+hGPR14 cells were lysed in a 4 M guanidium chloride buffer and total RNA was isolated using the RNeasy kit (Qiagen, Hilden, Germany). cDNA was synthesized by reverse transcriptase (Promega, Heidelberg, Germany) and subjected

Table 1. General characteristics of used cells. Mean $\pm$ SEM, n is given in brackets

	[mV]	V_m	ΔK^+	Ba ²⁺	Inh. PLA ₂
HEK293 -WT		-59 $\pm$ 3 (20)	13 $\pm$ 2 (20)	12 $\pm$ 2 (5)	2 $\pm$ 0.4 (5)
HEK293+hGPR14		-55 $\pm$ 1 (80)	13 $\pm$ 1 (79)	16 $\pm$ 2 (19)	6 $\pm$ 1 (15)
CHO -WT		-58 $\pm$ 3 (6)	16 $\pm$ 2 (6)		
CHO+hGPR14		-56 $\pm$ 2 (32)	15 $\pm$ 1 (32)	15 $\pm$ 2 (9)	-6 $\pm$ 2 (3)

to PCR reactions. For detecting mRNA for GPR14 the primers 5'-CTGGTGCTGGGCATCGTGCT-3' and 5'-AGGTGGTCECGGTAGTCC-3' (product size of 203 bp, annealing temperature of 64°C) were used. For detecting mRNA for GPR14 in mouse nephron segments the primers 5'-CCT ATT GGC TAT CCC AGA AA-3' and 5'-AGC AAG AAG CTA CCG TGG AA-3' (product size of 851 bp, annealing temperature of 64°C) were used. GAPDH expression was used as a positive control. PCR reaction products were analyzed by agarose gel electrophoresis and verified by sequence analysis (Sequence Laboratories, Göttingen, Germany).

Western blot

Whole kidneys from male C57BL mice were homogenized in 25 mM Tris-HCl, 1 mM NaVO₃, 1 mM EDTA, 1% SDS, and protease inhibitors. HEK293 cells transfected with the human GPR14 receptor were solubilized with the same solution to which 15% (w/v) Triton X-100 was added. Proteins were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a PVDF membrane incubated with blocking agent (Amersham, Freiburg, Germany). A goat polyclonal antibody directed against the C-terminal part of the ROMK channel was used (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Peroxidase-conjugated anti-goat secondary antibody was used and visualized by super signal chemiluminescent detection (Perbio Science, Bonn, Germany).

Fluorescence measurements of intracellular Ca²⁺

To measure the intracellular Ca²⁺ concentrations of HEK293 cells, the Ca²⁺-sensitive dye fura 2 was used [24]. The cells were incubated with the acetomethyl ester (fura2-AM; 5 μ M) dissolved with 0.1 g/l pluronic F-127 in standard solution for 45 min at 37°C in the dark and excited at 340, 360, and 380 nm. Fluorescence was detected at 500 to 530 nm with a single photon-counting-tube (H3460-04; Hamamatsu, Herrsching, Germany). The ratio of the emissions after excitations at 340 and 380 nm was calculated. The signal at 360 nm was used as Ca²⁺ independent control.

Biochemical Reagents

MEM was obtained from Gibco, human GN and UGN from Peptide Institute (Osaka, Japan), and AAC OCF₃ was purchased from Biomol (Hamburg, Germany), all other chemicals from Merck (Darmstadt, Germany) or Sigma (Taufkirchen, Germany).

Statistical Analyses

Student's unpaired *t*-test was used when effects between transfected cells and WT cells were compared. When the effects between more than two groups were tested, ANOVA with Tukey

as post test was used. Data are presented as mean values $\pm$ SEM. The number of experiments is given in brackets. *P* < 0.05 was set as significance level.

Results

General characteristics of used cells

HEK293 wild type (WT) cells were used from passages 24 to 44, 4-8 days after trypsination. HEK293 cells transfected with the human GPR14 receptor (HEK293+hGPR14) were used from passages 14 to 26, 2-11 days after trypsination. Basal membrane voltages (V_m), depolarizations caused by increasing the extracellular K⁺ concentration by 15 mM (ΔK^+), effects of Ba²⁺ (1 mM) or the inhibitor of PLA₂ (1 μ M) were not significantly different between WT and HEK293+hGPR14 cells (Table 1).

CHO WT cells were used from passages 8 to 14, 4-6 days after trypsination. CHO cells transfected with hGPR14 (CHO+hGPR14) were used from passages 9 to 15, 4-6 days after trypsination. Basal V_m and depolarizations caused by increasing the extracellular K⁺ concentration were not significantly different between WT and CHO+hGPR14 cells (table 1). In CHO+hGPR14 cells the inhibitor of PLA₂ (1 μ M) interestingly caused hyperpolarizations while the same inhibitor caused depolarizations in HEK293+hGPR14 cells, suggesting different downstream targets between the two expression systems.

Effects of guanylin peptides and urotensin II on V_m

To test if GPR14 is a possible receptor for guanylin peptides we used two cell types transfected with hGPR14. Guanylin peptides (10 nM each) and urotensin II (50 pM), a well known agonist for GPR14 [6], caused significantly higher depolarizations in HEK293+hGPR14 cells than in HEK293 WT cells (figure 1A). In contrast in CHO+hGPR14 cells guanylin peptides did not significantly increase depolarizations also observed in CHO WT cells. Compared to guanylin peptides, urotensin II (U-II) caused different effects in CHO cells, as it depolarized WT and

Fig. 1. Effects of guanylin peptides and urotensin II on HEK293-WT and HEK293+hGPR14 cells (A) and CHO-WT and CHO+hGPR14 cells (B). GN, UGN, and U-II caused higher depolarizations of HEK293 cells transfected with hGPR14 compared to WT cells. However, depolarizations caused by guanylin peptides were not different in CHO-WT and CHO+hGPR14 cells. The number of experiments is given in brackets (* $p < 0.05$ between effects in HEK293+hGPR14 compared to HEK293-WT cells). Schemes show the cells used and part of the signaling pathways examined.

hyperpolarized transfected cells (figure 1B).

This different behavior of guanylin peptides and U-II in HEK293+hGPR14 and CHO+hGPR14 cells could be due to different expression of proteins involved in GPR14 down stream signaling. Therefore, we used two GPR14 inhibitors: BIM-23127 [20] and urantide [21]. The effects of UGN and U-II (10 nM) in HEK293+hGPR14 and CHO+hGPR14 cells were inhibited by BIM-23127 (1 μ M) confirming the activation of the GPR14 receptor by guanylin peptides and U-II (figure 2). However, urantide (0.3 and 1 μ M), did not inhibit the UGN effects in HEK293+hGPR14 cells but still inhibited the U-II ef-

fects in these cells.

Guanylin peptides modified the membrane potential of mouse and human principal cells of cortical collecting ducts [14, 15] and proximal tubules by changing the K^+ conductance [13]. In HEK293+hGPR14 cells depolarizations caused by guanylin peptides or U-II were inhibited by the K^+ channel blocker Ba^{2+} (1 mM) (figure 3). However, hyperpolarizations caused by U-II (10 nM) in CHO+hGPR14 cells were not affected by Ba^{2+} (Figure 3B), suggesting again a different expression of proteins in both cell lines involved in GPR14 down stream signaling. A possible K^+ channel responsible for these

Fig. 2. Effects of GPR14 specific inhibitors on uroguanylin and urotensin II effects in HEK293+hGPR14 (A) and CHO+hGPR14 cells (B). BIM-23127 inhibited the effects of U-II in both cell types as well as UGN in HEK293+hGPR14 cells. Urantide inhibited only the U-II effects. The number of experiments is given in brackets (* $p < 0.05$ between effects in HEK293+hGPR14 cells with or without inhibitors). Schemes show the cells used and part of the signaling pathways examined.

effects of guanylin peptides or U-II on HEK-GPR14 cells is the ROMK channel, as this channel was already suggested as target for guanylin peptides in cortical collecting ducts [14, 15]. It is known that ROMK channels are present in HEK293 wild type cells [25]. Using western blot analysis we were able to show the presence of ROMK channels also in HEK293+hGPR14 cells used in this study. Lysates of mouse whole kidney were used as positive control for ROMK expression (figure 4).

Involvement of PLA_2 in signaling pathways

To test if ROMK and arachidonic acid are involved in the signaling pathway of guanylin peptides and U-II, we used AACOCF₃, an inhibitor of PLA_2 . In

HEK293+hGPR14 cells, AACOCF₃ (1 μ M) inhibited depolarizations caused by UGN (10 nM) or U-II (50 pM). The same inhibitor at 5 μ M reversed the depolarizations caused by urotensin II to hyperpolarizations (figure 5). These results suggest the existence of two signaling pathways for U-II in HEK293+hGPR14 cells. One causes depolarizations and is PLA_2 dependent, the other pathway leads to hyperpolarizations. In CHO+hGPR14 cells apparently only one signaling pathway exists. Hyperpolarizations caused by U-II were neither blocked by Ba²⁺, nor by AACOCF₃ (1 μ M), suggesting that this signaling pathway in CHO+hGPR14 cells does not involve K⁺ channels or PLA_2 , opposite to the U-II signaling pathways in HEK293+hGPR14 cells.

Fig. 3. Effects of Ba^{2+} on the effects of guanylin peptides and urotensin II in HEK293+hGPR14 cells (A) and CHO+hGPR14 cells (B). The number of experiments is given in brackets (* $p < 0.05$ between effects in HEK+hGPR14 cells in the presence or absence of Ba^{2+}). Schemes show the cells used and part of the signaling pathways examined. $K^+ch = K^+$ channels.

Fig. 4. Western blot analysis of ROMK channels: A=HEK293+hGPR14 cells; B=mouse kidney; C=rat kidney. A goat polyclonal antibody directed against the C-terminal part of the ROMK channel was used. Mouse whole kidney lysates were used as positive controls.

Effects of guanylin peptides and urotensin II on Ca^{2+} concentration

Urotensin II increases Ca^{2+} concentrations in CHO+hGPR14 [17] and HEK293+hGPR14 cells [16]. In this study only U-II (10 nM), but not GN and UGN (each 10 nM) increased intracellular Ca^{2+} concentrations

Fig. 5. Effects of the PLA₂ inhibitor AACOCF₃ on the change of V_m in HEK293+hGPR14 cells (A) and CHO+hGPR14 cells (B) induced by uroguanylin or urotensin II. The PLA₂ inhibitor at low concentrations (1 M) completely inhibited depolarizations caused by UGN and U-II. At higher concentrations, U-II led to hyperpolarizations of HEK+GRP14 cells. AACOCF₃ had no influence on hyperpolarizations of CHO+hGPR14 cells caused by U-II. The number of experiments is given in brackets (* $p < 0.05$ between effects of hormones in experiments with or without PLA₂ inhibitor). Schemes show the cells used and part of the signaling pathways examined. Inh – dep: inhibition of the potassium channels leads to depolarization; arach. acid: arachidonic acid. K⁺ch=K⁺ channels.

in HEK293+GPR14 cells. In these experiments ATP was used as positive control (figure 6).

To further investigate which part of the Ca²⁺ signaling pathway is involved in these cells we used thapsigargin, which inhibits Ca²⁺ release from stores, calphostin C, an inhibitor of PKC, and clotrimazol, an inhibitor of Ca²⁺-dependent K⁺ channels (figure 7). Depolarizations of HEK+hGPR14 cells caused by U-II did neither depend on the release of Ca²⁺ from intracellular stores nor on activation of PKC. On the other hand, after inhibition of PLA₂, hyperpolarizations caused by U-II were inhibited by Ba²⁺ and involved a release of Ca²⁺ from intracellular stores and PKC activity, but did not involve activation of

Ca²⁺ activated K⁺ channels. Hyperpolarizations of CHO+hGPR14 cells by U-II were inhibited in the presence of thapsigargin but not by inhibition of protein kinase C by calphostine C, which includes Ca²⁺ but excludes PKC involvement in the signaling pathway of U-II in CHO cells.

To understand why guanylin peptides did not increase Ca²⁺ but caused higher depolarizations in HEK293+hGPR14 cells compared to WT cells, we compared the concentration – response curves of voltage and Ca²⁺ effects induced by U-II as the only peptide which increased intracellular Ca²⁺ (figure 8). The inhibition of K⁺ channels, which causes depolarizations of the cells,

Fig. 6. Effects of guanylin peptides or urotensin II on intracellular Ca^{2+} concentrations. U-II but not GN and UGN changed intracellular Ca^{2+} concentrations in HEK+hGPR14 cells. ATP was used as positive control. The number of experiments is given in brackets.

Fig. 7. Effects of inhibition of the Ca^{2+} signaling pathway on depolarizations (A) and hyperpolarizations (B) of HEK+hGPR14 cells and (C) CHO+hGPR14 cells caused by urotensin II. Depolarizations of HEK+hGPR14 cells caused by U-II did not depend on release of Ca^{2+} from intracellular stores or activation of protein kinase C. On the other hand, hyperpolarizations caused by U-II were inhibited by Ba^{2+} and involved Ca^{2+} store release (thapsigargin) or activation of protein kinase C (calphostin C), but did not involve activation of Ca^{2+} dependent K^{+} channels (clotrimazol). U-II signaling pathway in CHO-hGPR14 cells involved Ca^{2+} as second messenger but not protein kinase C. The number of experiments is given in brackets (* $p < 0.05$ between effects of hormones in experiments with or without inhibitors). Schemes show the cells used and part of the signaling pathways examined. Inh – dep: inhibition of the potassium channels leads to depolarization; arach. acid: arachidonic acid; act – hyp: activation of potassium channels leads to hyperpolarization. K^{+} ch = K^{+} channels.

was caused by far lower concentrations of U-II ($\text{EC}_{50} = 70$ pM) compared to the concentrations needed for changes in intracellular Ca^{2+} concentrations ($\text{EC}_{50} = 30$ nM). These results suggest that guanylin peptides only increased arachidonic acid which altered V_m , while U-II at the same concentration led to changes in V_m via arachidonic acid and increases of the Ca^{2+} concentration.

Expression of GPR14 receptor

Expression of mRNA for the GPR14 receptor was detected using RT PCR. The GPR14 receptor was expressed in mouse proximal tubules and cortical collecting ducts (CCD) and in human kidney cortex and human CCD (figure 9). HEK293+hGPR14 cells were used as a positive control.

Fig. 8. Concentration-response curves of urotensin II effects on V_m and intracellular Ca^{2+} concentrations of HEK+hGPR14 cells. U-II depolarized cells at concentrations 400 times lower than those necessary to cause changes in Ca^{2+} concentrations. The number of experiments is given in brackets.

Fig. 9. Expression of GPR14 in mouse (A) and human (B) kidney. A: M = marker, 1=glomeruli, 2=proximal tubules, 3=cortical thick ascending limbs, 4=cortical collecting ducts, 5=neg. control (no cDNA), 6=pos. control (GAPDH). B: M=marker, 1=kidney cortex, 2=cortical collecting ducts, 3=HEK293+hGPR14 cells, 4=neg. control (no cDNA), 5=pos. control (GAPDH).

Discussion

The signaling pathway for guanylin peptides in principal cells of human and mouse cortical collecting ducts involves an activation of PLA_2 and an increase of intracellular arachidonic acid [14, 15]. So far no data exist on the molecular identity of possible receptors for guanylin peptides besides the GC-C, although evidence for the existence of another receptor is available, especially from GC-C deficient mice. Since it is known that activation of the G-protein coupled receptor GPR14 also leads to acti-

vation of PLA_2 and to an increase of cellular arachidonic acid [7], we have investigated whether the GPR14 receptor, which is expressed in mouse and human CCD (figure 9), may be involved in the signaling pathway of guanylin peptides in the expression systems HEK293 and CHO cells stably transfected with the human GPR14 receptor.

Our results show that guanylin and uroguanylin depolarized transfected HEK293 cells significantly higher than wild type cells (figure 1). This depolarization of HEK293 cells caused by both peptides was due to

inhibition of a K^+ conductance as the effects of UGN and also U-II were inhibited by Ba^{2+} (figure 3). Since Ba^{2+} is a nonspecific K^+ channel blocker these data do not allow to identify the molecular identity of the K^+ channels involved. In the CCD the secretory K^+ channel ROMK, which is responsible for K^+ secretion, is inhibited by arachidonic acid [23] and is involved in the signaling of guanylin peptides [14, 15]. As ROMK channels are also expressed in HEK293 cells, GPR14 is also a likely candidate as target for guanylin peptides and U-II in this expression system of renal origin (figure 4). In contrast to HEK293+hGPR14 cells, CHO cells transfected with the same receptor showed no difference in the depolarizations caused by guanylin peptides compared to WT cells. While the effects of U-II in HEK293+hGPR14 cells led to depolarizations, it caused hyperpolarizations in CHO+hGPR14 cells, suggesting either a different signaling pathway or a different final target for U-II in HEK293 cells compared to CHO cells. U-II effects in CHO cells were also independent of K^+ channel activity, again in contrast to the effects of GN and UGN. Thus, In HEK293 cells, U-II, GN, and UGN seem to have activated the same signaling pathway, leading to electrogenic conductance effects while in CHO cells such an electrogenic effect cannot be seen. This would most likely be due to differences in the signaling cascade downstream of possible receptor activation.

To further clarify the difference in activation of GPR14 in the two expression systems, we used potential GPR14 inhibitors. Figure 2A shows that the UGN effects were significantly inhibited by BIM-23127 in HEK293+hGPR14 cells. However, the more recently described antagonist of GPR14 urantide only blocked the effects of U-II but not of UGN. Assuming that urantide is a more specific inhibitor of the GPR14 receptor, this would suggest that guanylin peptides activate a similar signaling pathway as U-II in HEK293 cells but this does not necessarily involve the GPR14 receptor. Alternatively, one would have to postulate different binding sites for U-II and guanylin peptides at this receptor, of which only the U-II binding site is inhibited by urantide. As expected both inhibitors diminished hyperpolarizations of CHO+hGPR14 cells caused by U-II (figure 2B). In CHO cells guanylin peptides did apparently not activate the GPR14 receptor, although it cannot be excluded that the receptor and the signaling cascade was activated by these hormones, however, this activation does not lead to changes in electrogenic transporters or ion channels (see also below).

As reported before, activation of GPR14 leads to

an increase in intracellular Ca^{2+} and activation of PLA_2 which leads to an increase in cellular arachidonic acid [19]. To test the involvement of arachidonic acid in the signaling pathway of the tested hormones in HEK293+hGPR14 cells, we used AACOCF₃, a selective inhibitor of PLA_2 [26]. Stimulation of PLA_2 sets free arachidonic acid from plasma membrane phospholipids. The depolarizations caused by UGN (10 nM) or U-II (50 pM) on HEK293+hGPR14 cells were indeed inhibited by AACOCF₃ (1 μ M). This suggests again the involvement of PLA_2 , arachidonic acid and consequently probably ROMK channels in the signaling pathway of U-II and UGN in these renal cells. Interestingly, at a concentration of 5 μ M AACOCF₃ reversed the depolarizations caused by U-II into hyperpolarizations. These results suggest the existence of two signaling pathways for U-II in HEK293+hGPR14 cells (figure 5A). The predominant effect is the PLA_2 -dependent depolarization while the hyperpolarization can only be unmasked when the other pathway is inhibited. The hyperpolarization is due to an activation of a K^+ conductance as it was also blocked by Ba^{2+} (figure 7B). AACOCF₃ had no influence on hyperpolarizations of CHO+hGPR14 cells caused by U-II (figure 5B) which again suggest a different mechanism of U-II downstream signaling in these two different expression systems transfected with the same receptor.

Furthermore U-II also increased intracellular Ca^{2+} in HEK293+hGPR14 cells [16]. Thus, we tested whether Ca^{2+} could be involved as second messenger in the PLA_2 -independent effects of guanylin peptides in transfected HEK293 cells. Thapsigargin, which inhibits the Ca^{2+} release from the endoplasmatic reticulum by blocking Ca^{2+} -ATPases, inhibited the hyperpolarizations caused by U-II, suggesting an involvement of this signaling pathway in the hyperpolarizations induced by U-II. As inhibition of PKC by calphostin C also inhibited the hyperpolarizations induced by U-II, we can conclude that this second effect of U-II involves a Ca^{2+} -increase and activation of PKC, which then leads to activation of a K^+ conductance. To identify the properties of this K^+ conductance clotrimazole, an antifungal agent that acts as a specific inhibitor of Ca^{2+} activated K^+ channels, was used. As clotrimazole did not inhibit these hyperpolarisations, Ca^{2+} activated K^+ channels are probably not involved (figure 7B). Unlike these complex signaling pathways in HEK293+hGPR14 cells the only signaling pathway apparently present in CHO+hGPR14 cells is Ca^{2+} -dependent but PKC independent (figure 7C).

In conclusion, in the renal expression system

HEK293 cells guanylin peptides depolarized cells by activating PLA₂ which increases the intracellular concentration of arachidonic acid and inhibits ROMK channels. Whether GPR14 is the only G-protein coupled receptor activated by guanylin peptides and leading to an activation of PLA₂ also in the renal collecting duct remains to be proven further. Urotensin II is still the most potent agonist of the GPR14 receptor. However, the signaling pathway for U-II in HEK293 cells and CHO cells expressing GPR14 is significantly different. In CHO cells a PLA2 dependent signaling pathway activated via GPR14

was not detected. The only pathway for urotensin II that exists in CHO cells is Ca²⁺ dependent.

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