Membrane guanylate cyclase C (GC-C) is the receptor for guanylin, uroguanylin, and heat-stable enterotoxin (STa) in the intestine. GC-C-deficient mice show resistance to STa in intestine but saluretic and diuretic effects of uroguanylin and STa are not disturbed. Here we describe the cellular effects of these peptides using immortalized human kidney epithelial (IHKE-1) cells with properties of the proximal tubule, analyzed with the slow-whole-cell patch clamp technique. Uroguanylin (10 or 100 nM) either hyperpolarized or depolarized membrane voltages ($V_m$). Guanylin and STa (both 10 or 100 nM), as well as 8-Br-cGMP (100 μM), depolarized $V_m$. All peptide effects were absent in the presence of 1 mM Ba$^{2+}$. Uroguanylin and guanylin changed $V_m$ pH dependently. Pertussis toxin (1 μg/ml, 24 h) inhibited hyperpolarizations caused by uroguanylin. Depolarizations caused by guanylin and uroguanylin were blocked by the tyrosine kinase inhibitor, genistein (10 μM). All three peptides increased cellular cGMP. mRNA for GC-C was detected in IHKE-1 cells and in isolated human proximal tubules. In IHKE-1 cells GC-C was also detected by immunostaining. These findings suggest that GC-C is probably the receptor for guanylin and STa. For uroguanylin two distinct signaling pathways exist in IHKE-1 cells, one involves GC-C and cGMP as second messenger, the other is cGMP-independent and connected to a pertussis toxin-sensitive G protein.

Guanylin (GN) and uroguanylin (UGN) are heat-stable peptides that regulate electrolyte and water transport in intestine and stimulate kaliuresis and natriuresis in the kidney. Membrane guanylate cyclase C (GC-C) is the main receptor for these peptides in the intestine. GC-C was first described as a receptor for heat-stable enterotoxin (STa) secreted by *Escherichia coli* known to cause secretory diarrhea (1–3). All three peptides act from the luminal side of intestinal epithelial cells increasing cellular cGMP, which activates a cGMP-dependent protein kinase (PKG-II) (4, 5). Activation of the cystic fibrosis transmembrane conductance regulator located in the luminal membrane via cGMP and PKG leads to changes in Cl$^{-}$ and HCO$_3^-$ transport (6–9).

GN and UGN are proposed to be intestinal natriuretic factors. Both peptides are detected in human plasma and the intestine is probably the main source of circulating levels (10–12). Although GN and UGN are both filtered in the kidney, UGN is mainly present in urine. GN is probably degraded by chymotrypsin in the glomeruli (13) or the proximal tubules (14) or removed from the tubular fluid along the proximal tubule by endocytosis (15). Another possible source of UGN found in the urine are renal tubular cells, because mRNA for UGN as well as GN are detected in the kidney and in isolated tubule fractions (16–18). High salt diets elevate UGN and cGMP levels in the urine. STa also elevates urinary cGMP, and therefore it is reasonable to believe that these peptides act via a guanylate cyclase receptor similar to the intestinal receptor GC-C (11).

GC-C deficient mice (GC-C$^-/-$) show resistance to STa in intestine. In the intestine of GC-C$^-/-$ mice 10% of the specific $^{125}$I-STa binding to receptor sites still remain, suggesting the existence of an additional receptor distinct from GC-C (9, 19). Again two populations of binding sites for STa in the intestine have been identified: high affinity receptors not coupled to the guanylate cyclase (only 5% of binding sites) and low affinity binding sites coupled to the guanylate cyclase (95% of binding sites) (20). Effects of STa mediated via a signaling pathway independent of GC-C have not been detected so far. In the kidney UGN- and STa-induced natriuresis and kaliuresis in GC-C$^-/-$ mice is not disturbed in vivo (21). This indicates the existence of at least one additional receptor distinct from the GC-C receptor also in the kidney.

Our study describes for the first time cellular actions of GN, UGN, and STa in human kidney, specifically IHKE-1 cells (22), analyzed with the slow-whole-cell patch clamp technique. mRNA of GC-C was detected in IHKE-1 cells, human kidney, and in isolated human proximal tubules. In IHKE-1 cells GC-C was also detected by monoclonal antibodies. This is the first study that shows UGN actions via a novel pertussis toxin (PT)-sensitive G protein-coupled signaling mechanism besides the GC-C activated cGMP-dependent pathway. This mecha-
nism might also be responsible for the GC-dependent action of these peptides in extrarenal tissues.

**EXPERIMENTAL PROCEDURES**

**Cell Culture—**IHKE-1 cells derived from the proximal tubule were cultured in Dulbecco’s modified Eagle’s medium and Ham’s F-12 medium (1:1) containing in addition: 15 mM Hepes (pH 7.3), 1.6 mM epidermal growth factor, 100 μg/ml hydrocortisone, 65 mM transferrin, 0.84 μg/ml insulin, 29 mM Na₂S₅O₃, 15 mM NaHCO₃, 4 mM l-Gln, 5 μl/liter Ciproflox 100 (Bayer, Leverkusen, Germany), and 1% fetal calf serum as described before (23, 24). Cells were maintained in an atmosphere of 8% CO₂, 92% air at 37 °C. IHKE-1 cells were used from passages 140, 8–3 days (average 6 days) after trypsinization (0.05% trypsin, 0.02% EDTA in Mg²⁺-, and Ca²⁺-free phosphate buffer). For patch clamp experiments IHKE-1 cells were grown on glass coverslips. LLC-PK₁ cells, passages 180, grown on glass coverslips were used. Cells were cultured in Dulbecco’s modified Eagle’s medium containing 1% fetal calf serum, 100 000 IU/liter penicillin, 0.1% l-glutetmycetin, and 1 mM l-Gln in an atmosphere of 5% CO₂, 95% air at 37 °C. Subculturing was done in the same way as described for IHKE-1 cells. The cells were used 10 days after trypsinization.

**Pertussis Toxin—**PT, which is derived from Bordetella pertussis and catalyzes the ADP-ribosylation of subunits of G proteins, was used to examine the involvement of G proteins. Monolayers of IHKE-1 cells were incubated with medium containing 1 μg/ml PT for 4 or 24 h before experiments were performed.

**cGMP Assays—**IHKE-1 cells were cultured to 80% confluency in 12-well plates. Cells were treated for 10 min with 500 μl/well culture medium and 1 μM 3-isobutyl-1-methylxanthine (IBMX), or with IBMX and genistein (100 μM) (pH 7.4). Cells were incubated for another 10 min with culture medium containing IBMX or IBMX and genistein, and UGN, GN, STa, or ANP (as positive control). Reactions were stopped by addition of 70% ice-cold ethanol (500 μl/well). The ethanol supernatant was evaporated, the sediment was resuspended in 150 μl of 50 mM Na⁺-acetate buffer (pH 6.0) and acetylated (25), and cGMP was measured with a specific radioimmunoassay.

**Immunohistological Segments and Reverse Transcription—**PCR Analysis of Selected Tubules and IHKE-1 Cells—Human nephron segments were isolated using the procedure described previously for rat and rabbit (26, 27) and recently modified for guinea pig and human kidney from healthy cortical kidney pieces of patients (with written consent) undergoing tumor nephrectomies (28). Selected tubules of a kidney from healthy cortical kidney pieces of patients (with written consent) undergoing tumor nephrectomies (28) were dissected into segments. IHKE-1 cells were used 10 days after trypsination.

**Cell Culture—**IHKE-1 cells cultured on coverslips were washed three times with PBS and mounted in Vectashield mounting medium (Vector Laboratories, Burlingame, CA, USA). Cells were visualized under a Zeiss fluorescence microscope using standard filters for fluorescein isothiocyanate and 4,6-diamidino-2-phenylindole at ×100 magnification. **Patch Clamp Studies—**Coverslips with IHKE-1 or LLC-PK₁ cells were mounted as the bottom of a perfusion chamber on an inverted microscope (Axiovert 10, Zeiss, Oberkochen, Germany). Membrane voltages (Vₘ) were measured with the slow-whole-cell patch clamp method (30). A modified Ringer solution was used for experiments containing: 145 mM NaCl, 1.6 mM KH₂PO₄, 0.4 mM KHCO₃, 5 mM d-glucose, 1 mM MgCl₂, 1.3 mM calcium gluconate (pH 7.4). All substances used in experiments were dissolved in Ringer solution. Experiments were performed at 37 °C with a bath perfusion rate of 10 ml/min. The filling solution for patch clamp pipettes contained: 95 mM potassium gluconate, 30 mM KCl, 4.8 mM Na₂HPO₄, 1.2 mM NaHCO₃, 5 mM d-glucose, 0.726 mM calcium gluconate, 1 mM EGTA, 1.034 mM MgCl₂, 1 mM ATP (pH 7.2). To this solution, 160 μM nystatin were added to permeabilize the membrane. The solution was sterile filtered before use. Patch pipettes had an input resistance of 8–12 MΩ. Vₛ was measured with a patch pipette amplifier (U.-F. Fro, Physiological Institute, University of Tübingen, Germany). UGN was in addition purchased from Peptide Institute (Osaka, Japan) (no differences in activity of UGN from these different suppliers were observed). Epidermal growth factor, PT, and STa were obtained from Calbiochem (Schwalbach, Germany). All other standard chemicals were supplied by Merck (Darmstadt, Germany) and Sigma (Taufkirchen, Germany).

**RESULTS**

**Effects of GUANYLIN Peptides in IHKE-1 Cells**—IHKE-1 cells were incubated with fluorescein isothiocyanate-tagged anti-mouse IgG (Invitrogen, New Delhi, India) diluted 1:100 in blocking buffer for 1 h at room temperature. Cells were then washed six times with PBS and then incubated with fluorescein isothiocyanate-tagged anti-mouse IgG (Invitrogen, New Delhi, India) diluted 1:100 in blocking buffer for 1 h at room temperature. Cells were then washed six times with PBS and then incubated with 4,6-diamidino-2-phenylindole (250 ng/ml) for 3 min to stain the DNA and then incubated with 1% 3,3’-diaminobenzidine and 25 mM sodium phosphate buffer, pH 7.2 (0.9% sodium chloride, PBS) and fixed in PBS containing 4% paraformaldehyde for 30 min. Cells were then washed and incubated in 2% bovine serum albumin and 0.1% Triton X-100 in PBS to block nonspecific sites and permeabilize, respectively, for 1 h at room temperature. Cells were washed and then incubated with 10 μg/ml mouse monoclonal antibody to GCC-4D7, monoclonal antibody to GCC-4D7.
Effects of Guanylin Peptides in IHKE-1 Cells

FIG. 1. Changes of $V_{m}$ (mV) as response to GN, STa, and UGN in IHKE-1 cells measured with the slow-whole-cell patch clamp technique. GN (▲) and STa (■) caused depolarizations and UGN (●) mainly hyperpolarizations. UGN at higher concentrations caused also depolarizations. Data are mean values ± S.E., the number of experiments are given in the bracket. *, $p < 0.05$ paired Student’s t test.

Blocker Ba$^{2+}$ (1 mM, Fig. 2), indicating the involvement of changes in K$^{+}$ conductances. Effects of all peptides were restored after Ba$^{2+}$ was washed out.

GN and UGN caused increases in cGMP and Cl$^{-}$ secretion in T84 cells in a pH-dependent manner. GN is more potent in alkaline, UGN in acidic pH (31). Therefore, we compared the effects of GN, UGN, and STa at pH 5.5, 7.4, and 8.0 (Fig. 3). Changing the extracellular pH from 7.4 to 8.0 hyperpolarized cells by $\pm 3.3 \pm 0.5$ mV ($n = 19$) and to pH 5.5 depolarized cells by $\pm 21.9 \pm 1.5$ mV ($n = 19$). This acidification led to an inactivation of K$^{+}$ channels in the apical membrane of rabbit proximal tubule cells (32) and most likely also in IHKE-1 cells. In our study Ba$^{2+}$ (1 mM) still depolarized $V_{m}$ by $\pm 2.0 \pm 0.4$ mV ($n = 8$) at pH 5.5. This effect was 60% lower than the effect of Ba$^{2+}$ on the same cells at pH 7.4 ($\pm 5.9 \pm 0.5$, $n = 8$). GN (10 nM) caused depolarizations at all pH values but it was significantly less potent at pH 5.5 compared with pH 7.4 (pH 5.5, $\pm 1.0 \pm 0.2$ mV; pH 7.4, $\pm 2.2 \pm 0.3$ mV; pH 8.0, $\pm 1.3 \pm 0.4$ mV, $n = 7$). STa (10 nM) caused similar depolarizations at all pH values (pH 5.5, $\pm 1.3 \pm 0.1$ mV; pH 7.4, $\pm 2.0 \pm 0.3$ mV; pH 8.0, $\pm 1.5 \pm 0.3$ mV, $n = 5$). In paired experiments UGN (10 nM) showed depolarizations at pH 5.5 ($\pm 2.4 \pm 0.4$ mV, $n = 10$), when pH was changed to 7.4, UGN showed either depolarizations (1.5 ± 0.4 mV, $n = 6$) or hyperpolarizations (-2.6 ± 0.6 mV, $n = 4$) and after changing pH to 8.0 UGN caused only hyperpolarizations (-2.1 ± 0.3 mV, $n = 10$).

Taken together, these data suggest the involvement of cGMP in the signaling pathway for GN, UGN, and STa causing depolarizations of IHKE-1 cells. Activation of PT-sensitive G proteins open K$^{+}$ channels in the plasma membrane which can cause hyperpolarizations (33, 34). Therefore, we incubated cells with $1 \mu$g/ml PT for 24 h. Basal $V_{m}$ in these cells was $\pm 35.1 \pm 0.7$ mV ($n = 14$). Increasing the extracellular K$^{+}$ concentration from 3.6 to 18.6 mM depolarized these cells by $\pm 3.8 \pm 0.3$ mV ($n = 10$). Ba$^{2+}$ (1 mM) caused depolarizations of $\pm 5.4 \pm 0.3$ mV ($n = 7$). Preincubation of IHKE-1 cells with PT had no effect on depolarizations caused by GN, UGN, or STa (each 10 nM). Hyperpolarizations caused by 1 nM UGN (control, $\pm 1.6 \pm 0.5$ mV, $n = 8$; PT, $\pm 0.6 \pm 0.4$ mV, $n = 4$) and by 10 nM (control, $\pm 2.4 \pm 0.5$ mV, $n = 5$; PT, $\pm 0.5 \pm 0.2$ mV, $n = 6$) were inhibited. When pH was set to 8.0, effects of UGN (10 nM) were even reversed to depolarizations (Fig. 4). Incubation of cells with PT for only 4 h also completely blocked UGN-induced hyperpolarizations ($n = 5$).

The kinase homology domain of guanylate cyclases including GC-C is tyrosine phosphorylated in the basal state. The role of this phosphorylation in the GC-C function is not known yet (35, 36). In our study, an inhibitor of tyrosine kinases, genistein (10 μM), was able to block all depolarizations of $V_{m}$ of IHKE-1 cells induced by GN ($n = 7$) or UGN ($n = 11$, each 10 nM). Hyperpolarizations induced by UGN ($n = 4$), however, were not significantly altered in the presence of genistein (Fig. 5).

Effects of GN, STa, and UGN on Cellular cGMP—Cells were preincubated for 10 min with IBMX (1 mM) and incubated with GN, UGN, or STa in the presence of IBMX for 10 min. Concentrations of cGMP were measured with a specific radioimmuno-

Fig. 2. Inhibition of GN, STa, and UGN (10 nM) effects on $V_{m}$ (mV) in IHKE-1 cells in the presence of Ba$^{2+}$ (1 mM). Ba$^{2+}$ is a blocker of K$^{+}$ channels. Effects of GN and both effects of UGN and STa (10 nM) were absent in the presence (●) of 1 mM Ba$^{2+}$. All experiments are paired. Data are mean values ± S.E. *, $p < 0.05$ between peptides effects with and without Ba$^{2+}$ presence (paired Student’s t test).

Fig. 3. Effects of GN, STa, and UGN on $V_{m}$ (mV) in IHKE-1 cells at different pH (5.5, 7.4 and 8.0). GN (●) and STa (■) caused depolarizations at all pH values tested (GN less potent at pH 5.5). UGN (●) (10 nM) showed depolarizations at pH 5.5, hyperpolarizations at pH 8.0 and both effects at pH 7.4. All experiments are paired. Data are mean ± S.E. *, $p < 0.05$ between effects tested between different pH values, the number of experiments are given in bracket (ANOVA with posthoc Tukey test).

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assay. Results are summarized in Table I. ANP, which was used as positive control, increased cGMP in IHKE-1 as shown before (23). STa, GN, and UGN (1 μM and 100 nM) stimulated cGMP accumulation in IHKE-1 cells but were less potent than ANP.

Effects of PT and Genistein on UGN-dependent cGMP Accumulation in IHKE-1 Cells—IHKE-1 cells were incubated with PT (1 μg/ml) for 4 h. Cells were incubated with UGN (1 μM) in the presence of IBMX. PT did not influence UGN-dependent cGMP accumulation in IHKE-1 cells (Table II). To determine the influence of genistein on cGMP accumulation caused by UGN we preincubated cells with IBMX (1 mM) and genistein (100 μM). UGN did not increase cGMP accumulation in the presence of genistein any more (Table II).

GC-C in IHKE-1 Cells, Human Kidney, and Isolated Human Proximal Tubules—Human nephron segments were isolated from cortical kidney pieces of patients undergoing tumor nephrectomies. mRNA for GC-C was detected by reverse transcriptase-PCR in IHKE-1 cells, human kidney, and isolated human proximal tubules (Fig. 6).

Expression of GC-C in IHKE-1 Cells—To establish GC-C expression in IHKE-1 cells, cells were subjected to immunostaining using a monoclonal antibody raised to the protein kinase-like domain of GC-C, GC-C:4D7 (29), and followed by fluorescein isothiocyanate-labeled anti-mouse IgG. Specific staining could be seen in IHKE-1 cells indicating GC-C expression. No staining was observed in cells treated with the same concentration of normal mouse IgG (Fig. 7).

DISCUSSION

GC-C is a receptor for GN and UGN in the intestine. GC-C was first described as a receptor predominant for STa (1–3). UGN and STa induce natriuresis, kaliuresis, and diuresis in the rat in vivo (13) and in the isolated perfused rat kidney (37, 38). Since UGN- and STa-induced natriuresis and kaliuresis in vivo are not inhibited in GC-C-deficient mice (21) another so far unknown signaling pathway for UGN, STa, and GN, distinct

![Graph A](https://via.placeholder.com/150)

**Fig. 4.** Effects of GN, STa, and UGN on \( \Delta V_m \) (mV) in PT-treated IHKE-1 cells. Effects of GN, UGN, and STa in IHKE-1 cells incubated with medium containing 1 μg/ml PT for 24 h (■) or with medium without PT (■) are shown. Data are mean ± S.E. The number of experiments are given in the bracket. A, effects of GN and STa (both 10 nM) were not abolished by PT. B, in control cells UGN (10 nM) showed depolarizations and hyperpolarizations at pH 7.4, and hyperpolarizations at pH 8.0. In PT-treated cells UGN at both pH values caused only depolarizations. *, \( p < 0.05 \) between effects was tested by unpaired Student’s t test.

![Graph B](https://via.placeholder.com/150)

**Fig. 5.** Genistein (10 μM) inhibits only depolarizations induced by GN and UGN. Effects of GN and UGN in absence (■) and presence of tyrosine kinase inhibitor, genistein (■) (10 μM) are shown. Depolarizations caused by GN and UGN (both 10 nM) were blocked by genistein. All experiments are paired. Data are mean ± S.E. *, \( p < 0.05 \) between peptides effects with and without genistein presence (paired Student’s t test).

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**TABLE I**

|        | Control | GN | STa | UGN | ANP |
|--------|---------|----|-----|-----|-----|
| cGMP (fM) |         |    |     |     |     |
| 70 ± 16 (10) | 139 ± 24 (10) | 128 ± 23 (12) | 145 ± 29 (8) | 307 ± 81 (11) |

**TABLE II**

| Pertussis toxin (1 μg/ml) | Genistein (100 μM) |
|--------------------------|-------------------|
| Control | UGN Control | UGN |
| cGMP (fM) |         |    |     |     |
| 82 ± 4 (3) | 137 ± 13 (4) | 119 ± 14 (3) | 103 ± 9 (5) |
from GC-C, has to be assumed in the kidney. Therefore, the aim was to search for functional effects of these peptides on the cellular level of the human kidney and identify their possible signaling pathways.

In this study we show that GN, UGN, and STa regulate ion conductances and consequently electrogenic electrolyte transport in IHKE-1 cells, a human proximal tubule cell line. IHKE-1 cells grown to confluence on glass coverslips polarize and develop apical microvilli (23). In this preparation peptide hormones predominantly reach the apical membrane suggesting that the effects of these peptides act via apical membrane receptors. GN and STa depolarized cells at concentrations between 1 and 100 nM. In 20% of cells STa (10 nM) caused hyperpolarizations. UGN only hyperpolarized V_m at lower concentrations and caused both effects at higher concentrations. Since hyperpolarizations and depolarizations were seen in the same monolayers and no morphological and functional evidences for different cell types exist, we have to assume that IHKE-1 cells can principally respond to these peptides via two receptor/signaling pathways. The existence of two pathways is further demonstrated by the pH dependence of the effects (see below). To exclude that these effects of UGN are not only specific for IHKE-1 cells but are typical for proximal tubule cells, we tested UGN also in LLC-PK1 cells. In this porcine proximal tubule cell line, UGN again showed both effects. The depolarizations induced by all three peptides were due to inhibition of a K<sup>+</sup> conductance as the effects were completely absent when the K<sup>+</sup> conductances were inhibited by Ba<sup>2+</sup>. A similar effect was described by us before for ANP in these cells (23), which inhibited a Ca<sup>2+</sup>-dependent K<sup>+</sup> channel directly via cGMP (39). 8-Br-cGMP depolarized IHKE-1 cells also in this study and the effects of GN, UGN, and STa on cellular cGMP contents were similar to the effects of ANP in this and the previous study (23). cGMP is the established second messenger after GC-C activation and the mRNA for GC-C could be detected in IHKE-1 cells as well as in human kidney and isolated human proximal tubules. GC-C was also detected in IHKE-1 cells by immunostaining using a monoclonal antibody, GC-C:4D7. Taken together, these findings strongly indicate that GN, UGN, and STa can activate GC-C in these human proximal tubule cells leading to an increase in the cellular cGMP content and a reduction in a K<sup>+</sup> conductance which is localized in the apical membrane (39).

At low concentrations and in a large number of experiments also at higher concentrations UGN hyperpolarized V_m which was due to an activation of a K<sup>+</sup> conductance as again these effects were absent in the presence of Ba<sup>2+</sup>. This observation together with the fact that addition of a membrane permeable cGMP analogue depolarized V_m indicates the existence of two different signaling pathways for UGN: one is cGMP-dependent and mediated via GC-C and the other pathway is cGMP independent and mediated via a so far unidentified receptor.

To further discriminate between these two signaling pathways we tested whether the effects of GN, UGN, and STa on V_m...
of IHKE-1 cells were pH dependent, as was demonstrated for the activation of GC-C in colonic cells (31). In T84 cells, stimulation of cGMP production via GC-C by UGN was more potent at pH 5.5 and by GN at pH 8.0. STa showed no pH dependence in these cells (31). In our study GN effects were smallest at pH 5.5. UGN showed only depolarizations at pH 5.5 which fits with the hypothesis that at this pH the affinity of UGN to GC-C is highest. When the affinity of UGN to this receptor was lowered by changing pH to 8.0 UGN only hyperpolarized cells, again supporting the hypothesis of a second GC-C independent pathway. At pH 7.4 UGN showed either depolarizations (at higher concentrations) or hyperpolarizations (already at lower concentrations) via GC-C or via a cGMP independent pathway, respectively. The type of the response to UGN probably depends on the relative expression of these two receptors in the examined cells. Since in paired experiments UGN caused depolarizations at pH 5.5 and hyperpolarizations at pH 8.0, both signaling pathways coexist in the same cells. This pH dependence of the effects especially for UGN in the IHKE-1 cells may be physiologically relevant within the kidney as the luminal pH of the effects for UGN in the IHKE-1 cells may be physiologically relevant within the kidney as the luminal pH of the effects for UGN in the IHKE-1 cells may be physiologically relevant within the kidney as the luminal pH of the effects for UGN in the IHKE-1 cells may be physiologically relevant within the kidney as the luminal pH becomes more acidic along the nephron and preliminary data indicate that UGN and GN also act in the collecting duct.2

The role of tyrosine phosphorylation of the kinase homology domain in GC-C may play a role in regulation of GC-C signaling (35, 36). For the guanylate cyclase activation of guanylate cyclase A and B (GC-A, GC-B), phosphorylation of the kinase homology domain appears to be essential for ligand-induced activation (for review, see Ref. 40). Therefore, we tested whether the effects on $V_m$ induced by GN or UGN can be prevented by the presence of the tyrosine kinase inhibitor genistein. In this study all depolarizations induced by these peptides, and UGN-dependent cGMP accumulation were completely blocked by genistein. This is in line with the hypothesis that phosphorylation of GC-C is necessary for its activation.

The involvement of GC-C and cGMP as second messenger in the signaling pathway for GN, UGN, and STa causing depolarizations of IHKE-1 cells appears straightforward. The respective components involved in UGN and probably also STa mediated hyperpolarizations in these cells are less clear. As shown before, activation of cAMP and increases in cellular Ca2+ in IHKE-1 cells do not induce hyperpolarizations excluding an involvement of PKA and PKC (23). In muscle, neuron and kidney PT-sensitive G protein stimulation activates K+ channels (33, 34). Crane et al. (41) failed to demonstrate an effect of PT on GC-C-mediated effect of STa in the intestine excluding the involvement of G proteins in this signaling cascade. Connections between PT-sensitive G proteins and GN or UGN were neither tested in intestine nor in kidney. Preincubation of IHKE-1 cells with PT (1 μg/ml) had no effect on depolarizations caused by GN, UGN, or STa nor on UGN-dependent cGMP accumulation, in line again with the negative findings for STa in the intestine. Hyperpolarizations caused by UGN at pH 7.4, however, were inhibited by PT. These results suggest that the second receptor activated by UGN and probably also by STa is connected to a PT-sensitive G protein.

Although our data so far indicate the involvement of two distinct receptors for UGN actions in IHKE-1 cells, the possibility of one receptor activating two different signaling pathways should be discussed. In the intestine, STa can bind to and activate GC-C with high affinity without activation of the guanylate cyclase (20). To activate the guanylate cyclase activity, guanylate cyclase receptors (GC-A and GC-B) have to be phosphorylated at the kinase homology domain. Dephosphorylation leads to desensitization of these receptors (40). GC-C is phosphorylated in the basal state and undergoes a STa-induced time-dependent switching in the activity in the intestine (20). Phosphorylation of GC-C could play a similar role in its activity (35, 36). However, a possible dephosphorylation of GC-C leading to inactivation of the guanylate cyclase and consecutive activation of a coupled G protein appears unlikely as this should influence the effects of all three peptides. Such a situation has been described for the juxtapamembrane domain of the insulin-like growth factor receptor which couples to a G protein (3, 42, 43). Further strong arguments against the involvement of only one receptor molecule in these two signaling pathways for GN, UGN, and STa are the following: UGN and STa still produce kaliuresis and natriuresis in GC-C-deficient mice (21), STa still binds in the intestine to about 10% of that in wild type mice (19), and GN, UGN, and STa still showed effects on $V_m$ in isolated cortical collecting ducts of GC-C-deficient mice.3 These observations in GC-C-deficient mice indicate that there exists probably also a GC-C independent pathway for these peptides in the distal nephron. Alternatively in addition to the cloned GC-C there may be another isoform involved in renal GN, UGN, and STa effects which is still present in GC-C-deficient mice and which may still be coupled to cGMP. A receptor which has a 92–95% identity in the catalytic domain but only 55–58% identity in ligand-binding domain (OK-GC) compared with rat, pig, and human GC-C, was demonstrated for the opossum kidney (44).

The magnitude of the effects of all three peptides on $V_m$ due to changes in the K+ conductances is probably higher in proximal tubules in vivo than in these cultured proximal tubule cells. In vivo the cells of the proximal tubule have a higher K+ conductance and therefore, a higher $V_m$ (45). A depolarization of the apical membrane of the proximal tubule cells, as seen with activation of the GC-C, will reduce the driving force for Na+, substrate, and water reabsorption in this nephron segment.

In this study we demonstrate for the first time cellular actions of GN, UGN, and STa in the human kidney, specifically a proximal tubule cell line. We propose two distinct receptors and signaling pathways for these peptides. GN, UGN, and STa inhibit a K+ conductance via activation of GC-C and increases in cellular cGMP. The second receptor is activated by UGN with a higher affinity and to some extent also by STa. This so far unknown receptor couples to a PT-sensitive G protein which leads to the activation of a K+ conductance. Changes in K+ conductances and consequently $V_m$ will influence the driving force for electrogenic electrolyte and substrate transport across the proximal tubule and thereby modify their urinary excretion (Fig. 8).

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