Coordinate upregulation of guanylin and uroguanylin expression by hypertonicity in HT29-18-N2 cells

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Steinbrecher, Kris A., Jeffrey A. Rudolph, Guangju Luo, and Mitchell B. Cohen. Coordinate upregulation of guanylin and uroguanylin expression by hypertonicity in HT29-18-N2 cells. Am J Physiol Cell Physiol 283: C1729–C1737, 2002.—Guanylin and uroguanylin are particulate guanylate cyclase-activating peptides that are secreted from the epithelia of the intestine, kidney, pancreas, and salivary gland. These peptides elicit chloride and bicarbonate secretion via the cystic fibrosis transmembrane conductance regulator. To test the hypothesis that hypertonicity mediates an increase in guanylin and uroguanylin mRNA, we subjected HT29-18-N2 to osmotic stress. Guanylin and uroguanylin RNA were increased substantially in the presence of hypertonicity but only with solutes that were relatively impermeable to the cell membrane. This hypertonicity-mediated increase was transcriptional and did not require protein synthesis. Herbimycin A and mitogen-activated protein kinase inhibitors SB-203580 and PD-98059 had no effect on basal or induced levels of guanylin or uroguanylin. Both staurosporine and prolonged exposure to phorbol ester reduced basal levels and completely blocked hypertonicity-related increases in guanylin or uroguanylin RNA. These data suggest that serine/threonine protein kinases, possibly protein kinase C (PKC), mediate the hypertonicity-associated increase in guanylin and uroguanylin RNA. We conclude that guanylin and uroguanylin are released in response to hypertonic stress and that regulation of these genes may be mediated by PKC isoforms.

GUANYLIN AND UROGUANYLIN are produced and secreted by intestinal epithelia. One or both ligands are released by kidney, pancreatic, and salivary epithelia, as well. They are produced as prohormones, and the carboxy terminal region of each is cleaved to release the active, receptor-binding ligand, which then binds the transmembrane receptor guanylate cyclase C (GC-C). Specifically, activation of GC-C by guanylin or uroguanylin results in elevated cGMP and increased cystic fibrosis transmembrane conductance regulator (CFTR) activity (6, 10). This epithelial signaling system is thought to mediate release of intestinal chloride and probably bicarbonate but may also have distant effects on other epithelial cells (14, 15). Specifically, guanylin and in particular uroguanylin may serve a hormonal function and form an endocrine axis between the intestine and the kidneys. These peptides may regulate renal electrolyte and water transport during periods of salt absorption (5, 13, 16, 22). We have previously shown that guanylin and uroguanylin are increased in a whole animal model of osmotic diarrhea and speculated that these genes are responsive to the increased intraluminal hypertonicity inherent to this model (29).

To determine more fully the factors that result in increased guanylin and uroguanylin expression, we identified a novel in vitro intestinal epithelial model system that expresses both guanylin and uroguanylin RNA. The HT29-18-N2 intestinal cell line represents a reductionist system that lends itself to addressing questions concerning guanylin and uroguanylin production during isotonic and hypertonic exposure. Here, we show that guanylin and uroguanylin RNA demonstrate a transcription-dependent increase in response to hypertonicity and that proguanylin protein secretion is also increased during hypertonic exposure. Similar to other genes responsive to hypertonicity, the compatible osmolyte betaine blocks the induction of guanylin or uroguanylin RNA. Studies to determine which signaling pathways influence both basal and induced RNA levels of these ligands suggest that serine/threonine protein kinase cascades are involved and that, specifically, PKC isoforms may mediate increases in levels of guanylin and uroguanylin RNA during osmotic stress.

EXPERIMENTAL PROCEDURES

Cell culture and reagents. HT29-18-N2 cells were a gift from Dr. Cynthia Sears of Johns Hopkins University. These cells are mucin-secreting human intestinal epithelial cells and have been previously shown to express guanylin RNA (18). The experiments described here were performed by using cells from passages 5 through 15 that were <2 days old.

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past confluence because the effects of hypertonicity were lost at subsequent time points. This cell line does not form resistive monolayers with tight junctions. HT29-18-N2 cells were grown at 37°C and 10% CO₂ in Dulbecco’s modified Eagle’s medium (DMEM) plus 10% fetal calf serum supplemented with 10 μg/ml penicillin, 50 IU/ml streptomycin, and 5 mg of human transferrin (Sigma-Aldrich, St. Louis, MO) per liter. Cells were seeded into 12-well plates at equal amounts. Sodium chloride, urea, and mannitol were all supplied by Fisher Scientific (St. Louis, MO). Lactose was obtained from ICN Biochemicals (Aurora, OH). All other reagents and pharmacologicals used in experimental protocols, unless otherwise stated, were supplied by Sigma-Aldrich.

Experiments using hypertonic medium were performed as follows. Medium was removed from confluent wells of HT29-18-N2 cells, and the cells were washed twice with warm phosphate-buffered saline (PBS). Serum-free DMEM medium, with or without hypertonic agents, was placed on the monolayers, and the plates were then incubated at 37°C/10% CO₂ for 24 h. Concentrated solutions of NaCl, urea, or mannitol were added to bring the experimental medium to target osmolality. Unless otherwise stated, experiments were performed using +50 mM NaCl (+100 mosmol/kgH₂O), resulting in hypertonic medium of a calculated 400 mosmol/kgH₂O. Aliquots of medium containing each hypertonic agent and control medium were measured for pH and did not differ from the accepted norm for HT29-18-N2 culture medium (~pH 7.4). After the 24-h incubation period, the medium was removed, placed in microcentrifuge tubes, and stored at −80°C until use as described below.

Cells within each well were quickly washed with ice-cold PBS and then processed in one of two ways. Cells were either placed in Trizol Reagent (Life Technologies, Gaithersburg, MD) for RNA extraction or in Tris-mannitol lysis buffer for protein extraction. RNA and protein extraction methods are described below. Several activators and inhibitors of cellular proteins were used. RNA transcription and protein translation were inhibited by using actinomycin D and cycloheximide, respectively. PKC was activated and depleted in these studies by using differing exposure times to phorbol 12-myristate 13-acetate (PMA). A broad range of kinases was blocked by using herbimycin A, a tyrosine phosphorylation inhibitor, or staurosporine, a serine/threonine kinase inhibitor. The mitogen-activated protein kinases p38 and p42/p44 (ERK) were blocked with SB-203580 and PD-98059, respectively. All drugs were placed on the cells 15 min before the introduction of hypertonic medium unless otherwise stated, and concentrations of each can be found in the text. All pharmacologicals were dissolved in the minimum amount of vehicle (water, methanol, or dimethylsulphoxide) as recommended by the supplier. Within each study, control medium contained the same amount of vehicle as was used in drug-containing medium, which, in all cases, was ≪0.2%.

Trypan blue assay. HT29-18-N2 cells that had been incubated with either serum-free control or serum-free hypertonic mannitol medium (~300 mosmol/kgH₂O water control vs. ~400 or ~500 mosmol/kgH₂O hypertonic medium) for 24 h were trypsinized, briefly centrifuged, and resuspended. Cells were then incubated with 0.4% trypan blue for 5 min. Two wells each of control and hypertonic medium were used to determine cell viability by counting total cells, as well as stained cells in duplicate for each well using a hemocytometer.

Northern analysis. Total RNA was extracted from cells by using Trizol reagent. Briefly, cells were washed with PBS and ice-cold Trizol reagent was placed in each well. The cell monolayer was scraped free, and well contents were moved to a microcentrifuge tube and thoroughly mixed. Total RNA was then extracted according to the manufacturer’s protocol and stored at −80°C. RNA (20 μg/lane for each sample) was separated by using 1.5% agarose/7% formaldehyde gels. After confirmation of equal amounts of RNA in each well by ultraviolet visualization of 18S ribosomal RNA, gels were blotted onto Magnacharge nylon membrane (Osmonics, Westborough, MA) by using standard capillary transfer methods. Human guanylin and uroguanylin cDNAs were kindly provided by Dr. Mark Currie (Monsanto, St. Louis, MO). An actin cDNA probe was a gift from James Lessard (Children’s Hospital Research Foundation, Cincinnati, OH). Guanylin, uroguanylin, and actin cDNAs were labeled with 32P-dCTP by using a commercially available random primer labeling system (Life Technologies). Probes were hybridized to filters as described previously (7, 34). Hybridization signals were visualized by using the Molecular Dynamics PhosphorImager system (Molecular Dynamics, Cambridge, MA).

Western analysis. Proguanylin-specific antiserum (Ab4696) was used by using standard protocols. Briefly, a portion of the human guanylin gene that encodes prohormone residues 18–115 was placed into the expression vector pET21B, and a recombinant antigen (guanylin prohormone with polyhistidine tag) was produced according to the manufacturer’s suggested protocol (Novagen, Madison, WI). This antigen was injected into New Zealand White rabbits, and human proguanylin-specific antiserum was extracted as per standard techniques. Antiserum 4696 recognizes the correctly sized band during Western analysis only in human tissues, e.g., jejunum, ileum, and colon, that are expected to produce guanylin prohormone.

Protein extraction from HT29-18-N2 cells was performed at the indicated time points as follows. Medium was removed from each well, and this “spent” medium was stored at ~80°C until analysis. Cells were quickly washed with ice-cold PBS twice before collection of cell monolayer. Ice-cold 2 mM Tris-HCl/50 mM mannitol buffer containing a protease inhibitor cocktail (Protease Inhibitor Cocktail III; Calbiochem, San Diego, CA) was placed on each well (0.5 ml), and the cell monolayer was scraped free and placed in a glass dounce. After complete homogenization with the dounce, each sample was placed in a 1.5-ml centrifuge tube and spun at 16,000 g at 4°C for 20 min. The supernatant was then moved to a new tube and stored at ~80°C. Cell supernatant (40 μg) or spent medium (5 μl) from each sample was electrophoresed by using NuPAGE 4–12% gradient polyacrylamide gels and electrophobled onto nitrocellulose membranes according to the manufacturer’s protocol (Invitrogen, Carlsbad, CA). Membranes were immunoblotted by using proguanylin-specific antibody 4696–4 at a 1:1,000 dilution. After incubation with a secondary antibody conjugated to horseradish peroxidase, signal was visualized on Kodak X-OMAT AR film by using a commercially available chemiluminescence kit (NEN Life Science Products, Boston, MA).

Statistical analysis. All values are presented as means ± SE. Unless otherwise stated, all comparisons are made between cells in control isotonic DMEM for 24 h and cells in hypertonic medium using the unpaired t-test or analysis of variance (ANOVA) where appropriate. Differences were considered statistically significant at P < 0.05.

RESULTS

Hypertonicity increases guanylin and uroguanylin RNA. To determine the effect of hypertonicity on guanylin and uroguanylin RNA levels in HT29-18-N2 cells, hypertonic medium was placed on cells for 24 h

AJP-Cell Physiol • VOL 283 • DECEMBER 2002 • www.ajpcell.org
and RNA was extracted. Medium was made hypertonic with mannitol or sodium chloride to levels approximately +50 or +100 mosmol/kgH₂O above isotonicity (350 or 400 mosmol/kgH₂O vs. 300 mosmol/kgH₂O control). Guanylin and uroguanylin RNA were increased substantially in cells exposed to hypertonic medium of both +50 and +100 mosmol/kgH₂O for 24 h (Fig. 1, A–D). These increases were dose dependent with a maximal increase seen at +100 mosmol/kgH₂O using either mannitol or sodium chloride as the extracellular osmolyte. Use of more substantial hypertonicity (up to +300 mosmol/kgH₂O mannitol) did not further increase guanylin or uroguanylin RNA past that seen with +100 mosmol/kgH₂O (data not shown). In a parallel set of experiments, HT29 cells were exposed to +100 mosmol/kgH₂O mannitol for 24 h (Fig. 1E). RNA was prepared and probed for guanylin, uroguanylin, actin, and GAPDH. When normalized for GAPDH expression (which was unchanged) in the presence of mannitol, guanylin mRNA was increased 50% (P < 0.0001), uroguanylin mRNA was increased 52% (P < 0.0001), and actin mRNA was decreased 33% (P = 0.03).

Guanylin and uroguanylin RNA levels were first elevated at 8 h and remained elevated for 24–48 h after exposure to hypertonic DMEM (Fig. 2, A and B). In the experiment depicted in Fig. 2, basal levels of guanylin and uroguanylin decreased after 16 h. Levels of guanylin/uroguanylin consistently declined postconfluence. When preconfluent cells (70–80% confluent) were cultured for 24–48 h to achieve confluence, the decline in guanylin and uroguanylin levels was not seen between time zero and 16 h.

Cell death due to osmotic stress would influence guanylin and uroguanylin levels. Several studies have determined that HT29-18-N2 cells are viable at hypertonicity levels of ~500 mosmol/kgH₂O and higher (1, 21). To confirm this finding, cells exposed to +100 mosmol/kgH₂O or +200 mosmol/kgH₂O mannitol for 24 h were stained with 0.4% trypan blue to determine cell viability. No significant difference in cell viability between control and hypertonic medium was seen (95.3 ± 2.1% living control cells vs. 93.3 ± 2.7% +100 mosmol/kgH₂O mannitol and 93.1 ± 1.2% +200 mosmol/kgH₂O mannitol).

Having established that RNA of both genes is elevated after exposure to an increase of 50 mosmol/kgH₂O above isotonic levels, we next sought to identify whether these genes respond in a solute‐specific manner. We incubated HT29-18-N2 cells for 24 h with +100 mosmol/kgH₂O of NaCl, mannitol, lactose, or urea. Guanylin and uroguanylin RNA induction was

![Fig. 1. Dose-dependent induction of guanylin and uroguanylin RNA during hypertonic stress. Guanylin (A, B) and uroguanylin (C, D) RNA are elevated after a 24-h incubation with medium made hypertonic with 50 mosmol/kgH₂O or 100 mosmol/kgH₂O of mannitol or sodium chloride. Insets represent typical Northern blots at top and 18S ribosomal RNA at bottom to demonstrate equal loading. All values are expressed as means ± SE. Values for control (isotonic) medium were set to 100 for each experiment, and all others were adjusted accordingly, n = 4 per group. ANOVA for A: P < 0.0001; for B: P = 0.02; for C: P < 0.0001; for D: P = 0.003. Asterisks indicate significance vs. control (P < 0.05). E: 50% increased expression of guanylin and uroguanylin (P < 0.0001) and 33% decreased expression of actin after incubation of HT29 cells with 100 mosmol/kgH₂O of mannitol for 24 h.](http://ajpcell.physiology.org/)

AJP-Cell Physiol • VOL 283 • DECEMBER 2002 • www.ajpcell.org
dependent on a transmembrane osmotic gradient (Fig. 3, A and B). Both ionic (NaCl) and non-ionic (mannitol and lactose) solutes that are not readily membrane permeable caused increases in guanylin and uroguanylin levels. However, urea, a reagent that moves across the cell membrane, does not increase RNA for either gene and elicits a decrease in guanylin RNA. Of note, urea has been shown to negatively regulate the osmotically responsive gene aldose reductase, as well (30). Thus guanylin and uroguanylin are increased in response to the osmotic gradient created by impermeable solutes and not simply to hyperosmolality.

*Proguanylin production and secretion are elevated after exposure to hypertonicity.* We next sought to determine whether increases in guanylin and uroguanylin RNA during osmotic stress were reflected in increased prohormone production and secretion. Toward this end, the availability of a human proguanylin-specific antibody, Ab4696, allowed for the determination of cellular and secreted proguanylin levels in response to hypertonic conditions. Initial studies to assess the feasibility of the HT29-18-N2 cell line for use in determining proguanylin protein levels were performed. Proguanylin levels could be easily detected in newly confluent cells and in medium that had been bathed confluent cells for as little as 4 h (data not shown). Hypertonic medium (+100 mosmol/kgH2O NaCl) was placed on HT29-18-N2 cells for 24 h, and levels of proguanylin in cell homogenates and in medium were determined. Proguanylin levels found in HT29-18-N2 cells were increased 23% in response to hypertonic medium (Fig. 4A; P = 0.01). In addition, 41% more proguanylin hormone was released from HT29-18-N2 cells after 24 h in hypertonic medium compared with cells in control isotonic DMEM (Fig. 4B; P = 0.0005). Elevated levels of proguanylin in cell supernatant and medium that resulted from exposure to hypertonic conditions were of similar magnitude to changes in RNA levels (Fig. 1, A and B). This suggests that RNA levels of guanylin are increased to support elevated production and export of guanylin prohormone. Similar studies to assess the response of human prouroguanylin to osmotic stress will begin upon availability of prouroguanylin-specific antiserum.

*Guanylin and uroguanylin RNA transcription is increased during osmotic shock, and protein synthesis is not required for induction.* Next, we addressed the mechanism of the increase in RNA levels of guanylin dependent on a transmembrane osmotic gradient (Fig. 3, A and B). Both ionic (NaCl) and non-ionic (mannitol and lactose) solutes that are not readily membrane permeable caused increases in guanylin and uroguanylin levels. However, urea, a reagent that moves across the cell membrane, does not increase RNA for either gene and elicits a decrease in guanylin RNA. Of note, urea has been shown to negatively regulate the osmotically responsive gene aldose reductase, as well (30). Thus guanylin and uroguanylin are increased in response to the osmotic gradient created by impermeable solutes and not simply to hyperosmolality.

**Fig. 2.** Time course of guanylin and uroguanylin RNA increase during hypertonic stress. Guanylin (A) and uroguanylin (B) RNA are elevated beginning 8 h after exposure to medium made hypertonic with 100 mosmol/kgH2O of mannitol. All values are expressed as means ± SE. Values for control (isotonic) medium were set to 100 for each experiment, and all others were adjusted accordingly. In this experiment, cells were plated at confluence and levels of guanylin and uroguanylin declined in postconfluent culture; n = 3 per group. ANOVA for A: P < 0.008; for B: P = 0.03, with 8 h being different by Bonferroni correction in both groups. Asterisks indicate P < 0.05 vs. control without Bonferroni correction.

**Fig. 3.** Hypertonicity, not hyperosmolality, causes increased guanylin and uroguanylin RNA. Guanylin (A) and uroguanylin (B) respond to extracellular solutes that are relatively non-membrane permeable. NaCl, mannitol, and lactose cause elevated guanylin and uroguanylin levels, but the membrane-permeable urea does not. This implies the need for a transmembrane osmotic gradient for the guanylin and uroguanylin RNA response. All values are expressed as means ± SE. Values for control (isotonic) medium were set to 100 for each experiment, and all others were adjusted accordingly; n = 4 to 6 per group. ANOVA for A: P < 0.0001; for B: P < 0.0001. Asterisks indicate significance vs. control (P < 0.05).
and uroguanylin. To determine whether elevations in guanylin and uroguanylin RNA were due to increased transcription vs. increased RNA transcript stability, HT29-18-N2 cells were exposed to hypertonicity for 24 h. Medium was then replaced with hypertonic DMEM containing 5 \mu g/ml actinomycin D (1). Samples were taken at 6 h after exposure to actinomycin D. This approach allowed for the assessment of sustained increases in uroguanylin or guanylin after impairment of new transcript generation. A decrease was noted in the levels of guanylin after 6 h with actinomycin D, suggesting that RNA stability was not sufficient to result in the hypertonicity-induced elevation. Similarly, hypertonicity-induced uroguanylin expression was also decreased in the presence of actinomycin D, albeit not to basal levels. (Fig. 5). RNA from control wells containing hypertonic medium and methanol (actinomycin D vehicle) did not change during the 6-h time course for guanylin but was lower for uroguanylin. Although further characterization is necessary, this study suggests that guanylin and uroguanylin RNA levels are increased in response to hypertonicity through transcriptional means.

To determine whether protein translation was necessary to mediate hypertonic induction of guanylin and uroguanylin, we used cycloheximide to block protein synthesis in cells exposed to hypertonic medium. Cycloheximide (6.0 \mu M) in isotonic medium was placed on control and experimental cells 2 h before study initiation. At the start of the study, the preincubation medium was removed and fresh control and hypertonic medium containing 6.0 \mu M cycloheximide was placed on the cells for 24 h. Control and hypertonic medium samples were increased 2.5- to 5-fold compared with samples not exposed to cycloheximide (Fig. 6, A and B). Even in the presence of cycloheximide, however, a consistent increase in guanylin and uroguanylin levels was seen during osmotic stress. This finding suggests

![Image](https://i.imgur.com/330x159.jpg)

**Fig. 4.** Cellular and secreted proguanylin protein is increased after exposure to hypertonicity. Proguanylin-specific antibody 4696 (Ab4696), which recognizes the ~12-kDa prohormone, was used to determine both cellular and secreted levels of proguanylin. Cellular levels of proguanylin (A) and secreted proguanylin found in cell culture medium (B) are significantly higher after exposure to hypertonicity (+100 mosmol/kg H2O NaCl). Typical blots from 4 to 6 experiments per group are shown.

![Image](https://i.imgur.com/77x600.jpg)

**Fig. 5.** Effects of actinomycin D on basal and hypertonicity-induced guanylin and uroguanylin RNA levels. Elevated levels of guanylin and uroguanylin RNA are not maintained after 6 h of 5.0 \mu g/ml actinomycin D (Act. D). All values are expressed as means ± SE. Values for control (isotonic) medium were set to 100 for each experiment, and all others were adjusted accordingly; n = 4 per group. a indicates significance vs. isotonic untreated control (P < 0.05); b indicates significance vs. hypertonic untreated (P < 0.05).

![Image](https://i.imgur.com/68x141.jpg)

**Fig. 6.** Cycloheximide treatment does not block osmotic stress-mediated RNA increases in guanylin and uroguanylin. The presence of cycloheximide has no effect on guanylin (A) and uroguanylin (B) induction during exposure to hypertonicity. Basal levels of expression are significantly increased during exposure to cycloheximide. All values are expressed as means ± SE. Values for control (isotonic) medium were set to 100 for each experiment, and all others were adjusted accordingly; n = 5 to 6 per group. a indicates significance vs. isotonic untreated control (P < 0.05); b indicates significance vs. cycloheximide-treated control (P < 0.05).
that the transcriptional response of these genes to hypertonicity is not dependent on protein synthesis. The increases in guanylin and uroguanylin mRNA seen after cycloheximide treatment, in the presence or absence of hypertonicity, could be consistent with the loss of a normally present inhibitor that represses guanylin/uroguanylin transcription.

The compatible osmolyte, betaine, blocks guanylin and uroguanylin RNA induction during hypertonic stress. To establish the induction of guanylin and uroguanylin as a response to hypertonicity and its effects on cellular function, betaine was added to both control and hypertonic medium. This provides the HT29-18-N2 cell layer with a compatible osmolyte with which to counter the hypertonic effects of the NaCl gradient on the cell exterior. Although addition of 5.0 mM betaine to control medium did not significantly change the level of guanylin or uroguanylin RNA, addition of betaine blocked the expected increase in guanylin or uroguanylin after 24 h in hypertonic medium (Fig. 7, A and B). The lack of guanylin or uroguanylin RNA elevation in hypertonic medium containing betaine is similar to that seen in other osmotically responsive genes (12, 28).

Serine/threonine protein kinase pathways modulate guanylin and uroguanylin RNA levels in isotonic and hypertonic conditions. Both basal and hypertonicity-induced levels of guanylin (A) and uroguanylin (B) RNA are unaffected by exposure to herbimycin A, indicating little or no role for tyrosine kinase-dependent pathways in regulating this process. However, staurosporine significantly decreased both isotonic and hypertonicity-induced RNA levels of both genes, suggesting that serine/threonine protein kinase pathways are relevant to guanylin and uroguanylin transcript regulation and stress response. All values are expressed as means ± SE. Values for control (isotonic) medium were set to 100 for each experiment, and all others were adjusted accordingly; n = 6 per group. a indicates significance vs. isotonic untreated control (P < 0.05); b indicates significance vs. herbimycin A-treated control (P < 0.05); c indicates significance vs. hypertonic untreated group (P < 0.05).

Serine/threonine protein kinase pathways regulate guanylin and uroguanylin responses to hypertonic shock. To identify the intracellular signaling pathways that regulated the induction of guanylin and uroguanylin during osmotic stress, we determined the response of these genes to hypertonic medium in the presence of broad-spectrum kinase inhibitors (Fig. 8). First, HT29-18-N2 cells were exposed to 1.0 μM herbimycin A (1) for 15 min before and during the 24-h exposure to +100 mosmol/kg H2O medium made hypertonic with additional NaCl. This tyrosine protein kinase inhibitor did not significantly alter either basal and hypertonicity-induced guanylin or uroguanylin levels (Fig. 8, A and B). Therefore, tyrosine protein kinase activity does not appear to have a significant role in regulating guanylin or uroguanylin RNA abundance. Second, staurosporine was used to block signal transduction through serine/threonine phosphoryla-
tion-dependent pathways. Addition of staurosporine (0.1 μM) resulted in a striking decrease in both basal and tonicity-activated guanylin and uroguanylin RNA transcripts (Fig. 8, A and B). These data implicate serine/threonine protein kinase pathways in regulation of both basal and induced levels of guanylin and uroguanylin RNA.

PKC is inhibited by staurosporine and represented a candidate-signaling kinase for further investigation. The phorbol ester PMA activates some PKC isoforms in the short term (minutes to a few hours) but eventually results in depletion of PKC activity through exhaustion of PKC stores (2, 24, 27, 31). PMA was used to deplete PKC levels in HT29-18-N2 cells bathed in isotonic or hypertonic NaCl-containing medium, and this resulted in a substantial decrease in guanylin and uroguanylin RNA (Fig. 9). We next asked whether guanylin and uroguanylin RNA would increase if PKC were stimulated. A short, 2-h exposure to 0.1 μM PMA in isotonic medium resulted in large increases in guanylin and uroguanylin RNA and suggests that PKC activity influences basal guanylin and uroguanylin RNA transcript levels (Fig. 10).

Because mitogen-activated protein kinases (MAPK) mediate some cellular responses to osmotic shock (23, 26, 33), we next determined the effect of inhibition of certain p38 isoforms and p42/p44 (ERK) signaling using the specific inhibitors SB-203580 and PD-98059 (9, 11). Inhibition of p38α and p38β activity with 10 μM SB-203580 or ERK activity with 10 μM PD-98059 had no effect on basal expression of guanylin or uroguanylin RNA and also did not change the magnitude of the guanylin and uroguanylin RNA increase after 24-h exposure to medium made hypertonic (+100 mosmol/kgH2O) with NaCl (Table 1). Although these data suggest no role for these extensively characterized MAPKs, the potential importance of other members of this family (e.g., p38δ) will need to be investigated (19, 32, 35).

**DISCUSSION**

Guanylin and uroguanylin are both expressed in the epithelia of the mammalian intestine. Guanylin is present primarily in the distal small intestine and colon, and uroguanylin is largely produced in the proximal small intestine. However, some overlap exists and it is presumed that both ligands have similar function(s). Very little is known about the physiological conditions that control guanylin and uroguanylin expression and the signal transduction pathways that mediate this regulation. Identification of an in vitro model of guanylin and uroguanylin expression would therefore be desirable. Of the many human and rodent intestinal cell lines that have been surveyed, most do not express either guanylin or uroguanylin (7, 18, 34). The human Caco-2 cell line expresses guanylin after postconfluenc differentiation but does not express uroguanylin. Several subclones of the HT29 tissue culture line exhibit low levels of guanylin RNA, but few.

**Table 1. Guanylin mRNA (% of control)**

| Inhibitor (or solvent vehicles) were placed on the cells 15 min before the introduction of hypertonic medium. Inhibition of p38α and p38β activity with 10 μM SB-203580 or ERK activity with 10 μM PD-98059 had no effect the magnitude of guanylin mRNA increase after 24 h exposure to medium made hypertonic (+100 mosmol/kgH2O) with NaCl. Guanylin signal was normalized to 18S RNA levels.

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<th>Medium</th>
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<td>171 ± 8</td>
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Fig. 9. Long-term treatment of HT29-18-N2 cells with phorbol ester results in loss of hypertonicity-mediated increases in guanylin and uroguanylin RNA. Similar to staurosporine treatment, 24-h exposure to phorbol 12-myristate 13-acetate (PMA) resulted in substantial decreases in basal levels of guanylin and uroguanylin RNA. PMA treatment also eliminates the inducing effect of osmotic shock. All values are expressed as means ± SE. Values for control (isotonic) medium were set to 100 for each experiment, and all others were adjusted accordingly; n = 6 to 12 per group; a indicates significance vs. isotonic untreated control (P < 0.05); b indicates significance vs. hypertonic untreated group (P < 0.05).
express uroguanylin at levels that are readily detectable by Northern analysis. Only the goblet cell-like HT29-18-N2 tissue culture line (a subclone of the human HT29 adenocarcinoma clone) expresses both guanylin and uroguanylin RNA. Both genes are robustly expressed before and at confluence. Here, we describe the coordinate transcriptional induction of guanylin and uroguanylin in response to extracellular hypertonicity. Overall, the response of guanylin and uroguanylin was qualitatively and quantitatively similar. Furthermore, we present the first demonstration that serine/threonine protein kinase pathways may mediate both basal and osmotic stress-mediated increases in guanylin and uroguanylin.

We have previously reported induction of guanylin and uroguanylin in a mouse model of osmotic diarrhea (29). After 48 h of lactose-rich chow or 40 mM polyethylene glycol-containing drinking water, guanylin and uroguanylin RNA and prohormone levels were increased substantially in intestinal epithelia, leading us to speculate that persistent hypertonic intestinal contents mediated this increase. Consistent with these animal studies, the data presented here. Increases in guanylin and uroguanylin RNA are similar in magnitude to that seen in vivo. These data are also consistent with the physiological observations that salt loading increases guanylin levels (20) and that uroguanylin may serve as a mechanism for the intestine to alert the kidney to high salt intake (14, 15, 22). However, we have demonstrated that hypertonic conditions other than salt can increase guanylin and uroguanylin to a similar degree. The physiological significance of these stimuli remains to be investigated.

Treatment with either ionic or non-ionic solutes that did not easily cross the cell membrane resulted in increases in guanylin and uroguanylin RNA despite the broadly negative effects of cell shrinkage associated with hypertonicity (17). Urea, however, is relatively membrane permeable and the hyperosmolality that it generates did not cause increases in guanylin and uroguanylin RNA levels. These differences suggest that the presence of an osmotic gradient across the cell membrane is necessary for guanylin and uroguanylin mRNA induction. Moreover, the regulation of these genes by toxicity and not simply osmolality suggests that guanylin and uroguanylin are regulated by changes in intracellular ion concentration, cytoskeletal reorganization, and/or other membrane perturbations. Further experimentation involving cytoskeletal stabilizing agents and ion channel inhibitors is planned to distinguish among these possibilities.

In Madin-Darby canine kidney (MDCK) epithelial cells, external hypertonicity may be rectified by transport of betaine to levels 1,000-fold above that found in medium (36). The supplementation of compatible osmolytes, i.e., betaine, into hypertonic medium greatly diminishes the transcriptional response of two important osmotically controlled genes, aldose reductase and the betaine transporter (12, 28). Similarly, we found that including betaine in the medium of HT29-18-N2 cells that are exposed to toxicity of +100 mosmol/kgH2O eliminates the increase in guanylin and uroguanylin RNA. This provides additional evidence that these genes are tightly controlled by intestinal epithelial cell osmoregulation.

Several osmotic stress-activated kinase cascades are known to facilitate transcriptional increases, stabilization or degradation of RNA, increased protein translation, and activation or inhibition of functional proteins (3, 4, 17). The serine/threonine protein kinase inhibitor staurosporine caused a decrease in both basal level of guanylin and uroguanylin RNA, as well as a diminished response to hypertonicity. Extended PMA treatment had a similar effect on guanylin and uroguanylin RNA. PKC activity is regulated by hypertonicity but is blocked by both staurosporine and long-term exposure to phorbol esters (1, 37). Initial studies suggest that by using highly specific inhibitors of PKC such as bisindolylmaleimide, basal and induced RNA levels of guanylin and uroguanylin are almost totally blocked (data not shown). We also report here that activation of PKC through short-term exposure to PMA increases guanylin and uroguanylin RNA. Although the MAPKs p38 and ERK are activated in many cell types by changes in hypertonicity, the use of specific inhibitors of these kinases did not affect guanylin and uroguanylin basal expression or toxicity-induced elevation in HT29-18-N2 cells.

Collectively, these data support several conclusions. First, the identification of serine/threonine protein kinase pathways in the regulation of guanylin and uroguanylin levels represents a novel association between specific intracellular signaling networks and the RNA abundance of these genes. Second, PKC is a likely candidate for the regulation of guanylin/uroguanylin RNA levels at both basal and hypertonicity-stimulated levels in HT29-18-N2 cells. Because PMA results in a relatively rapid increase in guanylin/uroguanylin mRNA and the induction of guanylin/uroguanylin by hypertonicity requires a longer incubation, it is possible that other serine/threonine kinases are involved or that other signal transduction mechanisms subsequent to PKC activation are required for hypertonicity-mediated changes. It was recently reported that PKC positively influences transcription of the guanylin/uroguanylin receptor, GC-C, and that the activity of this receptor is upregulated by PKC-mediated phosphorylation (8, 25, 31). Taken together, these data suggest that PKC may act at several points in the guanylin/uroguanylin-GC-C-signaling pathway, perhaps in response to hypertonicity caused by high levels of extracellular solutes. Further work to determine which specific PKC isoforms are involved, what transcription factors are activated, whether cytoskeletal reorganization is a constituent factor in this process, and what other serine/threonine protein kinases may influence these genes will provide a more complete picture of how intracellular signaling networks control guanylin and uroguanylin expression.

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