Urea Signaling in Cultured Murine Inner Medullary Collecting Duct (mIMCD3) Cells Involves Protein Kinase C, Inositol 1,4,5-Trisphosphate (IP_3), and a Putative Receptor Tyrosine Kinase

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Abstract

Urea, in concentrations unique to the renal medulla, increases transcription and protein expression of several immediate–early genes (IEGs) including the zinc finger-containing transcription factor, *Egr-1*. In the present study, the proximal 1.2 kb of the murine *Egr-1* 5′-flanking sequence conferred urea-responsiveness to a heterologous luciferase reporter gene when transiently transfected into renal medullary mIMCD3 cells, and this effect was comparable with that of the extremely potent immediate-early gene inducer, *O*-tetradecanoylphorbol 13-acetate (TPA). Urea inducibility of *Egr-1* expression was protein kinase C (PKC)-dependent because staurosporine and calphostin C abrogated the urea effect, and down-regulation of PKC through chronic TPA treatment inhibited both urea-inducible *Egr-1* protein expression and gene transcription. In addition, hyperosmotic urea increased inositol 1,4,5-trisphosphate (IP_3) release from mIMCD3 cells and induced tyrosine phosphorylation of the receptor tyrosine kinase-specific phospholipase C (PLC) isoform, PLC-γ. Importantly, urea-inducible *Egr-1* expression was strongly genistein-sensitive, to a much greater extent than the comparable TPA-inducible *Egr-1* expression. These data suggest that urea-inducible *Egr-1* expression is a consequence of sequential PLC-γ activation, IP_3 release, and PKC activation. Urea-inducible PLC-γ activation, in conjunction with the genistein-sensitivity of urea-inducible *Egr-1* expression suggest the possibility of a cell surface or cytoplasmic urea-sensing receptor tyrosine kinase. (*J. Clin. Invest.* 1996. 97:1884–1889.) Key words: kidney • cell culture • hyperosmolarity • signal transduction • transcription factor

Introduction

Cells of the mammalian renal medulla are exposed to markedly elevated concentrations of NaCl and urea as a consequence of the renal concentrating mechanism. In cultured renal epithelial cells, hyperosmotic NaCl upregulates transcription and expression of several proteins responsible for mediating adaptation to a hypertonic milieu including the enzyme aldose reductase (1), as well as the Na^+/betaine (2) and Na^+/*myo*inositol (3) cotransporters. A NaCl-responsive mitogen-activated protein kinase (MAPK)[1] signaling pathway has recently been described in renal epithelial cells (4, 5). In addition, two distinct putative cell-surface sensors of hyperosmotic NaCl (and other impermeant solutes) have been identified in yeast (6, 7).

In marked contrast, the signaling events engendered by the other principal renal medullary solute, urea, have received less attention. In contrast to NaCl, stress induced by elevated and fluctuating urea concentrations occurs in mammals only in the renal medulla, and potentially systemically in the setting of advanced chronic renal insufficiency. Unlike NaCl, a functionally cell-membrane impermeant solute, urea is relatively membrane-permeant and has traditionally been considered to play a passive role in renal epithelial cell function (8). The ambient urea concentration in the mammalian renal medulla may exceed 1 M in some desert rodents, a concentration clearly associated with protein denaturation and competitive inhibition of enzyme activity (9).

Hyperosmotic urea increases transcription and expression of several immediate-early genes (IEGs), including the zinc finger-containing transcription factor, *Egr-1* (10, 11). *Egr-1* is inducible at the mRNA level by diverse hyperosmotic stressors (including NaCl), but at the protein level by only urea (12). *Egr-1* was originally described as a mitogen-responsive transcription factor (13–17); it binds to a unique DNA consensus sequence found upstream of diverse genes (18–20), of which only a small subset have thus far been demonstrated to confer functionality. *Egr-1* is the only eukaryotic gene known to be transcriptionally upregulated by hyperosmotic urea (12). The 5′-flanking sequence of the murine gene confers transcriptional responsiveness to a reporter gene in diverse contexts (21–24).

In the present study, transcription of *Egr-1* was used as a model system to investigate urea signaling to IEG expression. The proximal 1.2 kb of the murine *Egr-1* promoter was sufficient to confer urea-responsiveness to a luciferase reporter gene in transient transfection of renal medullary mIMCD3 cells, and in a protein kinase C (PKC)-dependent fashion. In addition, hyperosmotic urea augmented inositol 1,4,5-trisphos-

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1. *Abbreviations used in this paper:* EBS, Egr-1 binding site; IEG, immediate-early gene; IMCD, inner medullary collecting duct; IP_3, inositol 1,4,5-trisphosphate; MAPK, mitogen-activated protein kinase; mIMCD3, murine inner medullary collecting duct cell line; PKC, protein kinase C; PLC, phospholipase C; TPA, *O*-tetradecanoylphorbol 13-acetate.

phate (IP_3) release which appeared to be a consequence of enhanced tyrosine phosphorylation (and hence activation) of the receptor tyrosine kinase-specific phospholipase C (PLC) isoform, PLC-γ. Furthermore, this process appeared to be extremely genistein-sensitive, unlike *O*-tetradecanoylphorbol 13-acetate (TPA) signaling or previous reports of NaCl signaling (4, 5). Taken together, these data suggest the possibility of a cell surface (or cytoplasmic) receptor tyrosine kinase which functions as a urea receptor or sensor.

Methods

Cell culture. mIMCD3 cells (25) were maintained in DMEM/F12 supplemented with 10% FBS as described previously (26). Cells were growth-suppressed in DMEM/F12 without serum for 24 h before pretreatment for 30 min with staurosporine (100 or 500 nM), calphostin C (250 nM, in the presence of constant illumination), or genistein (100 μM, unless otherwise indicated). Medium was then supplemented with hyperosmotic solute to a final concentration of 200 mOsm (510 mOsm total). For PKC depletion, monolayers were treated with TPA (10^{-7} M) for 6 h before urea (200 mOsm) or TPA (10^{-7} M) treatment for 30 min.

IP_3 assay. Treated monolayers on 10 cm dishes were washed with ice-cold PBS, precipitated with 2 ml 4% perchloric acid, scraped from the dish into polypropylene tubes, incubated on ice × 20 min, and pelleted at 2000 *g* × 15 min at 4°C. Supernatants were retained and titrated to pH 7.5 with 60 mM Hepes, 1.5 M KOH. IP_3 levels were detected via a radioreceptor binding assay (Amersham), according to the manufacturer's directions.

Transient transfection. For transfection via electroporation, mIMCD3 cells were grown to 80–90% confluence, trypsinized, resuspended in warmed complete medium. They were then pelleted at 1,000 *g* × 5 min, washed with ice-cold DMEM/F12, re-pelleted, and resuspended in ice-cold DMEM/F12 at a working concentration of approximately 5×10^6 cells/ml. Cell suspension (0.5 ml) was added to 20 μg of *Egr-1* Luc reporter plasmid and 5 μg of the CMV-Gal vector (for normalization) in ice-cold electroporation cuvettes (Invitrogen), incubated on ice for 10 min, electroporated at 1000 μF and 300 V (GenePulser, BioRad), incubated on ice for 10 min, diluted 1:20 with warmed complete medium, and plated. *Egr-1* Luciferase reporter plasmid consisted of 1.2 kb of the murine *Egr-1* 5′-flanking sequence, excised as a SalI fragment from construct No. 632 (pEgr-1p1.2; kindly provided by V.P. Sukhatme, Beth Israel Hospital, Boston, MA), and ligated into the polylinker SalI site upstream of the luciferase reporter gene in the promoterless vector pXP2 (27). This construct includes all sequence data listed under EMBL accession number X12617 (22).

Reporter gene assays and EMSA. After 24 h, cells were placed in serum-free DMEM/F12; at 48 h, cells were treated for 6 h with the desired condition prior to harvest for determination of reporter gene activity. β-Galactosidase activity was determined using standard methods (28). To measure luciferase activity, individual wells of six-well plates were washed with ice-cold PBS and lysed with 150 μl of Luciferase Lysis Buffer (125 mM Tris, pH 7.6, 0.5% Triton X-100). Lysate (100 μl) was incubated with 200 μl of 5 mM ATP in Luciferase Buffer (25 mM glycylglycine, 15 mM $MgSO_4$, pH 7.8) and 100 μl of luciferin (60 μg/ml; Analytical Luminescence) in Luciferase Buffer in an automated luminometer (Berthold), counted for 30 s, and normalized to β-galactosidase activity. Data is presented as mean±SEM, except where noted; statistical comparison was achieved with ANOVA (StatView) wherein significance was ascribed to a $P < 0.05$ via Scheffe F-test, as previously described (29). EMSA for *Egr-1* DNA binding ability was performed as previously described (12).

Immunoprecipitation and Western analysis. Confluent monolayers were growth-suppressed in the absence of serum for 24 h before treatment with hyperosmotic solute. 10 min before treatment, monolayers received 1 mM sodium orthovanadate (pH 7.4). PLC-γ immunoprecipitation was performed in accordance with published methods (30, 31). At 5 min of treatment, monolayers were washed with ice-cold PBS and lysed for 30 min at 4°C with Lysis Buffer (1% Triton X-100, 10% glycerol, 50 mM NaF, 1 mM AEBSF, 1 mM sodium orthovanadate, 10 μg/ml leupeptin, in 20 mM Hepes, pH 7.2). Lysates were clarified at 14,000 *g* × 5 min at 4°C. Lysate from a single 10 cm dish was incubated with 5 μg of anti-PLC-γ antiserum (UBI) for 2 h at 4°C with constant gentle inversion. Thereafter, washed fixed *Staphylococcus aureus* (Calbiochem) was added and the incubation was continued with inversion for an additional 60 min. Immunoprecipitates were pelleted, washed ×4 with Wash Buffer (1% Triton X-100, 1% deoxycholate, 150 mM NaCl, 0.1% SDS, 1 mM sodium orthovanadate, in 50 mM Tris, pH 8.5), and raised in Laemmli sample buffer (28). Samples were boiled for 3 min and subjected to electrophoresis on a 10% sodium dodecyl sulfate/polyacrylamide gel electrophoresis. Proteins were transferred to polyvinylidine difluoride as described (12). Blots were probed with anti-phosphotyrosine antibody (UBI; 1:4000) or anti-PLC-γ antiserum (UBI; 1:10,000) according to the manufacturer's directions. Secondary antibody was goat anti-mouse monoclonal (BioRad) at 1:4000; detection was achieved with enhanced chemiluminescence (Amersham).

Results

When the proximal 1.2 kb of the murine *Egr-1* 5′-flanking sequence was cloned upstream of a luciferase reporter gene, the resulting construct exhibited urea-inducible luciferase activity upon transient transfection into mIMCD3 cells (Fig. 1). Luciferase activity in response to the potent *Egr-1* inducer, TPA, was comparable to that seen with urea, underscoring the magnitude of the urea response. TPA appeared to synergize with urea in enhancing *Egr-1* transcription. Not shown, when mIMCD3 cells were transiently transfected with a luciferase reporter gene driven by the (enhancerless) heterologous promoter, thymidine kinase, urea-inducibility was not observed.

Egr-1 induction in diverse contexts is dependent upon PKC activation. To assess the PKC-dependence of urea-inducible *Egr-1* transcription, several approaches were taken. mIMCD3 cells, transiently transfected with the *Egr-1*-Luc reporter gene, were pretreated with the PKC inhibitors, staurosporine or calphostin C. Pretreatment with relatively low and high doses of staurosporine suppressed reporter gene activity essentially to baseline, while exhibiting negligible effect upon control expression (Fig. 2). Pretreatment with calphostin C, in contrast, markedly inhibited both control and urea-inducible reporter gene activity (Fig. 2).

To confirm this result, prolonged treatment of mIMCD3

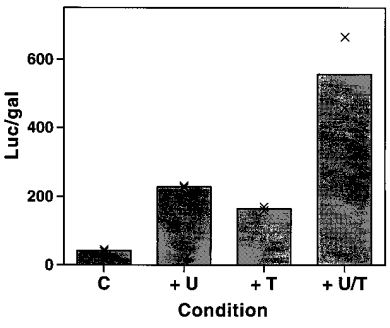

Figure 1. The proximal 1.2 kb of the murine *Egr-1* 5′ flanking sequence confers urea-responsiveness to a luciferase reporter gene. mIMCD3 cells, transiently transfected with 20 μg of *Egr-1*(1.2) Luc and 5 μg of CMV-Gal, were treated for 6 h with control medium (*C*), or medium supplemented with 200 mOsm urea (+ *U*), 10^{-7} M TPA (+ *T*), or urea + TPA (+ *U/T*). Bars represent mean luciferase activity, relative to β-galactosidase activity, for two wells; individual data points are depicted. Data are representative of three separate experiments.

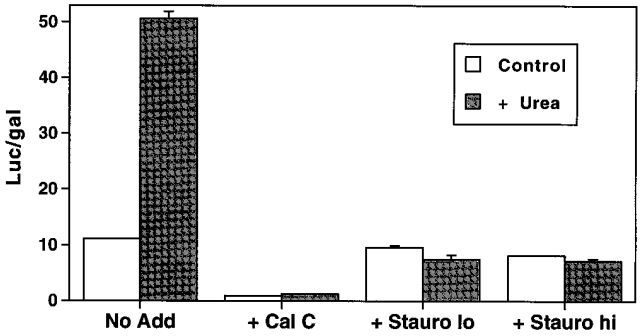

Figure 2. Urea-inducible *Egr-1* transcription is abrogated by inhibitors of PKC. mIMCD3 cells were transfected as in Fig. 1; before urea (200 mM) treatment, monolayers received 30 min of pretreatment with 100 nM (+ *Stauro lo*) or 500 nM (+ *Stauro hi*) staurosporine, or calphostin C (250 nM; + *Cal C*). Bars represent mean±SEM of at least three wells. Data are representative of two separate experiments.

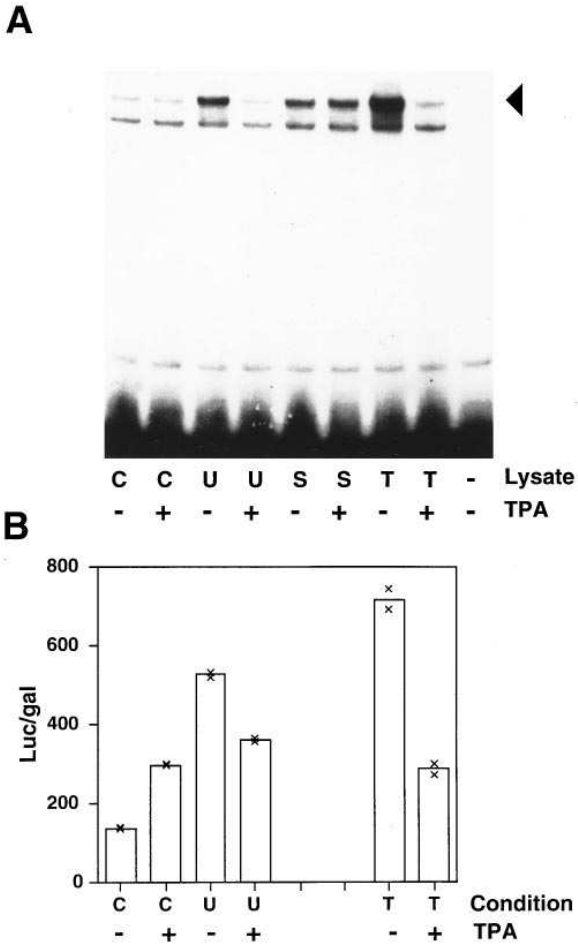

Figure 3. PKC down-regulation abrogates urea-inducible *Egr-1* expression. (*A*) EMSA of whole cell lysates prepared from mIMCD3 cells pretreated (+ TPA) or not pretreated (−TPA) with TPA (10^{-7} M) for 6 h before treatment for 30 min with control medium (*C*) or medium supplemented with 200 mM Urea (*U*), 20% FBS (*S*), or 10^{-7} M TPA (*T*). Probe was radiolabeled double-stranded oligonucleotide encoding the EBS consensus sequence to which *Egr-1* binds. The arrowhead indicates the specific *Egr-1*:EBS protein:DNA complex. (*B*) Luciferase reporter gene assay of mIMCD3 cells transiently transfected as in Fig. 1, pretreated (+) or not pretreated (−) with 10^{-7} M TPA for 6 h, before treatment for 6 h as described for *A*. In *B*, serum experiments were not performed. Bars are the mean of two wells; individual data points are depicted.

cells with TPA was used to determine the effect of PKC down-regulation upon urea-inducible *Egr-1* expression. Two complementary strategies were used. First, the effect of PKC down-regulation upon *Egr-1* protein expression in response to urea treatment was evaluated using the EMSA. This technique permits direct observation of protein:DNA interaction and has been previously shown to correlate with the degree of immunodetectable *Egr-1* protein in this model, in both the presence and absence of urea treatment (12). An end-labeled double-stranded oligonucleotide encoding the *Egr-1* binding site (EBS), the DNA consensus sequence with which the *Egr-1* protein interacts, was used. In the absence of lysate, no specifically retarded band was visible (Fig. 3 *A*). When control-treated cells were pre-treated with TPA (e.g., PKC down-regulation) for 6 h, there was no appreciable effect upon the basal low level of *Egr-1* protein expression (retarded upper band in Fig. 3 *A*). That this band, in fact, represents *Egr-1* protein has been previously confirmed in cold competition experiments utilizing EBS as well as irrelevant oligonucleotides (12). Prior PKC down-regulation, as would be expected, markedly suppressed acute TPA-inducible *Egr-1* expression. Similarly, PKC down-regulation abrogated *Egr-1* expression in response to hyperosmotic urea, implying the presence of a PKC-dependent mechanism. The serum effect, however, was not inhibited by PKC down-regulation.

In a related experiment, the effect of TPA-induced PKC down-regulation upon urea-inducible *Egr-1* transcription (as opposed to protein expression) was evaluated in mIMCD3 cells transiently transfected with the *Egr-1* Luc reporter gene construct (Fig. 3 *B*). At 6 h of TPA treatment, a modest increase in basal *Egr-1* expression was still detectable (Fig. 3 *B*), but this time point was employed for consistency with Fig. 3 *A*. As expected, PKC down-regulation potently suppressed acute TPA-inducible *Egr-1* expression to levels seen with control treatment. Similarly, and consistent with Fig. 3 *A*, PKC down-regulation also inhibited urea-inducible *Egr-1* transcription to the level seen under control conditions. Not shown, but also consistent with Fig. 3 *A*, serum-inducible reporter gene activity was not suppressed by PKC down-regulation.

As PKC activation is generally a consequence of PLC activation and concomitant IP_3 generation, the ability of hyperosmotic urea to stimulate IP_3 release in mIMCD3 cells was evaluated. By radioreceptor binding assay, hyperosmotic urea increased IP_3 release from mIMCD3 cells three-fold at 5 min of treatment (Fig. 4).

In general, PLC activation and IP_3 release are a consequence of activation of either a G-protein-coupled seven-transmembrane-spanning domain receptor or of a receptor tyrosine kinase. Preliminary data suggested that urea-inducible *Egr-1* expression was not G-protein mediated (data not shown). The effect was neither pertussis-toxin sensitive, nor inhibitable by overexpression of a dominant negative G_q in transient transfection. Therefore, the ability of the receptor tyrosine kinase inhibitor, genistein, to abrogate urea-inducible *Egr-1* expression was evaluated. mIMCD3 cells were transiently transfected with *Egr-1* luciferase reporter gene, pre-treated with

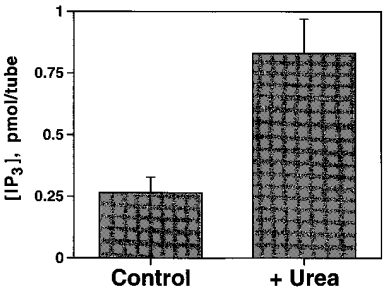

Figure 4. Urea treatment augments IP_3 generation. IP_3 content (expressed as pmol/assay tube) of control- and urea-treated mIMCD3 cells, as measured by radioreceptor assay. Bars are mean ±SEM of three separate wells. Results are representative of three separate experiments.

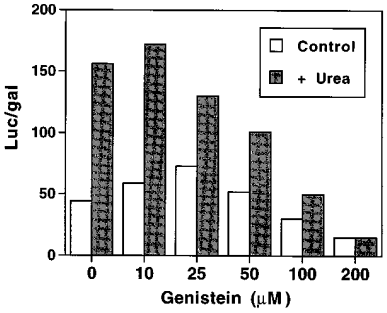

Figure 6. Dose-response of genistein-sensitivity of urea-inducible *Egr-1* transcription. Luciferase reporter gene assay of mIMCD3 cells transiently transfected as in Fig. 1, then pretreated with the indicated concentrations of genistein for 30 min before control (Control) or urea (+ Urea) treatment. Bars are the mean of two wells; depicted results are representative of two separate experiments.

genistein (100 μM), and evaluated for urea-inducible *Egr-1* transcription (Fig. 5). Genistein pre-treatment markedly inhibited urea-inducible *Egr-1* expression, and modestly inhibited the basal (Control) level of *Egr-1* transcription; control- and urea-inducible effect were suppressed to the same new baseline (Fig. 5). Importantly, genistein pre-treatment suppressed TPA-inducible *Egr-1* expression to a much lesser extent. The dose-response with which genistein inhibits urea-inducible *Egr-1* expression is shown in Fig. 6; 100 μM resulted in almost complete suppression with minimal effect upon basal expression, whereas 200 μM genistein suppressed both to an equal level below control.

With this indirect circumstantial evidence that urea-inducible *Egr-1* expression was mediated via a putative receptor tyrosine kinase, the ability of urea to upregulate activation (i.e., tyrosine phosphorylation) of the receptor tyrosine kinase-specific PLC isoform, PLC-γ, was investigated (32). Lysates were prepared from cells treated with control or urea-supplemented medium and then immunoprecipitated with anti-PLC-γ antiserum. After extensive washing, immunoprecipitates were resolved via SDS/PAGE and subjected to Western analysis with an anti-phosphotyrosine antiserum. Marked upregulation of tyrosine phosphorylation of a band migrating at ~ 130 kD was evident (Fig. 7 *A*). To confirm the identity of this band as PLC-γ, blots were stripped and reprobed with the original anti-PLC-γ antibody (Fig. 7 *B*). The upregulated band detected by anti-phosphotyrosine antiserum (Fig. 7 *A*) precisely co-migrated with the band detected by anti-PLC-γ (Fig. 7 *B*) and was of the expected molecular mass for PLC-γ (31). In the presence of urea treatment, only the degree of phosphorylation (but not the abundance) of immunodetectable PLC-γ was upregulated.

Discussion

These data show that the proximal 1.2 kb of the *Egr-1* 5′ flanking sequence confers urea-responsiveness to a heterologous (luciferase) reporter gene when transiently transfected into mIMCD3 cells. Urea-inducible *Egr-1* expression was mediated through a PKC-dependent process as evidenced by inhibitor studies and PKC down-regulation with chronic TPA exposure. In addition to activating PKC, hyperosmotic urea also increased IP_3 release in these cells, and upregulated phosphorylation of the receptor tyrosine kinase-specific PLC isoform, PLC-γ. Taken together, these data suggest that hyperosmotic urea mediates its effect upon IEG transcription through a signaling cascade initiated by a cell surface or cytoplasmic receptor tyrosine kinase. These are also the first studies to link activation of a specific signaling pathway to the "downstream" expression of a solute-inducible gene.

Two lines of evidence suggest the possibility of urea-inducible *Egr-1* expression initiated by a membrane-associated or cytoplasmic urea-sensing receptor tyrosine kinase. First, urea-inducible gene expression is extremely sensitive to the receptor tyrosine kinase inhibitor, genistein. However, several potential downstream signaling molecules (e.g., MAPK kinase) also require tyrosine phosphorylation for activation (33). Therefore, inhibitor sensitivity alone is insufficient to strongly support this contention. Both urea and TPA activate IEG transcription in a PKC-dependent fashion (Fig. 3); their respective signaling

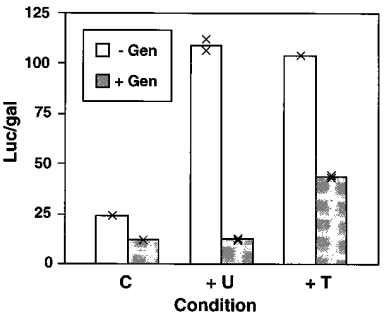

Figure 5. Urea-inducible *Egr-1* transcription is more sensitive to genistein than TPA-inducible *Egr-1* transcription. mIMCD3 cells were transfected as in Fig. 1 and then pretreated with 100 μM genistein (+ Gen) or sham-pretreated (−Gen) before treatment with control medium (*C*), urea (200 mM; +*U*), or TPA (10^{-7}; +*T*). Bars are the mean of two wells (individual data points are depicted); results shown are representative of two separate experiments.

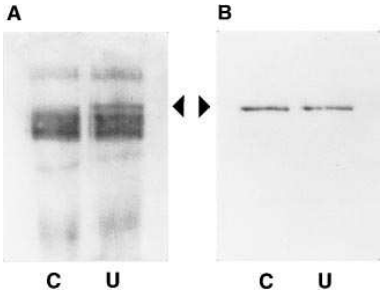

Figure 7. Urea treatment causes tyrosine phosphorylation of PLC-γ. Western analysis of whole cell lysates, prepared from monolayers subjected to control medium (*C*) or medium supplemented with urea (200 mM × 10 min). Depicted blot was probed first with anti-phosphotyrosine antibody (*A*), stripped and then probed with anti-PLC-γ antiserum (*B*). Arrowheads in each panel indicates PLC-γ.

pathways converge upon PKC. However, although urea activates *Egr-1* transcription (as measured by reporter gene activity in the transient transfection assay) to a roughly equivalent extent as the extremely potent immediate-early gene activator TPA, the former is much more sensitive to genistein than the latter. This suggests that the genistein-sensitivity of the urea effect is not entirely a consequence of inhibition of downstream kinases, and that there is a highly genistein-sensitive tyrosine phosphorylation event upstream of the point where the TPA and urea signaling pathways converge. Second, urea results in tyrosine phosphorylation of the receptor tyrosine kinase-specific PLC isoform, PLC-γ, an event associated with activation of PLC-γ (32). Taken together, these data strongly suggest the possibility that urea signaling is initiated by a urea-sensing cell surface or cytoplasmic tyrosine kinase. Whether the primary function of this putative kinase is to sense extracellular urea concentration, or whether this represents an ancillary function of a known or novel kinase remains to be shown. Such a receptor would possess extremely low affinity for its substrate because the half-maximal effect is in the range of 100 mM urea. The parathyroid calcium sensor affords a precedent for receptor:ligand interaction with a K_m in the mM range (34).

Although the presence of a urea-sensing tyrosine kinase has not been proposed elsewhere, ample precedent exists for a solute-sensing receptor. Two distinct cell surface tonicity (NaCl) sensors were cloned from the yeast, *S. cerevisiae* (6, 7). One of these, SSK1, is a histidine kinase which exhibits homology with a tonicity sensor operative in prokaryotes (6). In response to changes in ambient tonicity, these sensors trigger a cascade of kinase signaling events, culminating in the phosphorylation and concomitant activation of the osmotically responsive yeast MAPK homologue, HOG1 (6, 7, 35). A sensor of impermeant solute concentration has yet to be identified in higher eukaryotes.

Sensitivity to genistein and the concomitant phosphorylation of PLC-γ distinguish urea signaling from that of NaCl in renal medullary cells. Terada (5) and Itoh (4) observed a NaCl-inducible cascade of kinase signaling events in the canine renal epithelial MDCK cell line. NaCl treatment sequentially activated Raf, MEK, and an ERK-like MAPK. MAPK activation occurred in a PKC-dependent fashion (4, 5); however, it was insensitive to genistein treatment (5). These data, in conjunction with those presented here, strongly suggest that urea and the functionally impermeant solutes (e.g., NaCl) signal through divergent pathways. Consistent with such a model, physiological responses in these two contexts are distinct. Cell membrane-impermeant solutes such as NaCl cause an acute decrement in cell volume, whereas readily membrane-permeant solutes such as urea do not (8). In addition, dissimilar patterns of organic osmolyte accumulation are observed in response to osmotic challenge with either urea or the functionally impermeant solutes (8). Furthermore, whereas hyperosmotic urea increases *Egr-1* mRNA and protein expression, hyperosmotic NaCl actually suppresses *Egr-1* expression at the protein level (12), presumably as a consequence of a global inhibition in protein synthesis (29). It is reasonable to conclude that such dissimilar physiological responses would be activated by unique molecular triggers. It was not possible to evaluate adequately the effect of NaCl (in contrast to urea) in the present studies because the effect of hyperosmotic NaCl on *Egr-1* transcription and promoter activity is negligible compared with that of urea (data not shown).

Inducible expression of *Egr-1* may occur in a PKC-dependent fashion as observed here in response to hyperosmotic urea, or in a PKC-independent fashion. PKC-dependent models of *Egr-1* upregulation in fibroblasts have included induction by TPA (36), IGF-I (37), and inflammatory cytokines (38), but not by serum (36, 37), EGF (36), v-src (39), activated v-Fps (40), or heavy metals (41). Additional examples of PKC-dependence include induction of *Egr-1* by lipopolysaccharide (but not by granulocyte-macrophage colony stimulating factor) in peritoneal macrophages (42, 43); induction by platelet-derived growth factor or 5HT (but not by AVP) in glomerular mesangial cells (44); induction by X-irradiation in epithelial tumors (45); and induction by anti-receptor antibody in B lymphocytes (46). Many of these examples of PKC-dependent *Egr-1* expression occur in the setting of mitogen treatment. The renal epithelial cell response to hyperosmotic urea exhibits several hallmarks of mitogenesis; however, cell number is not increased (47).

Several details of the assays used to address the PKC-dependence of urea-inducible *Egr-1* expression warrant comment. Whereas staurosporine suppressed urea-inducible *Egr-1* transcription to essentially control levels, calphostin C suppressed both to below control values. Whether this is a consequence of a more potent PKC-inhibitory effect of calphostin C impinging upon a PKC-dependent component of basal *Egr-1* expression, or whether calphostin C is inhibiting a different signaling pathway is unclear. The former interpretation is suggested by the purported greater specificity of calphostin C and by the observation that mIMCD3 cells fail to completely growth-arrest despite serum starvation, and hence exhibit basal growth-associated kinase activity (data not shown). Suppression of *Egr-1* protein expression by PKC down-regulation (in the EMSA in Fig. 3 *A*) was more complete than the suppression of *Egr-1* reporter gene activity by PKC down-regulation (Fig. 3 *B*). This is likely a consequence of the difference in half-lives between the short-lived *Egr-1* protein (48) and the longer-lived luciferase protein. Urea-inducible *Egr-1* reporter gene activity (transcription) was greater than that inducible by TPA (Fig. 3 *B*), whereas urea-inducible *Egr-1* protein expression is less than that inducible by TPA (Fig. 3 *A*). This is likely a consequence of additional non-transcriptional regulatory mechanisms known to affect IEG mRNA and protein half-life, and potentially IEG protein post-translational modification.

These data, in conjunction with those of others, imply a renal medullary urea signaling pathway distinct from that associated with impermeant solutes. Additional work will be required to clarify the role of a putative urea-sensing tyrosine kinase in initiating the cellular response to hyperosmotic urea in vitro, and potentially, in mediating physiological adaptive processes in response to fluctuating urea concentrations in the renal medulla in vivo.

Acknowledgments

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