PI3K signaling in the murine kidney inner medullary cell response to urea

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Zhang, Zheng, Xiao-Yan Yang, Stephen P. Soltoff, and David M. Cohen. PI3K signaling in the murine kidney inner medullary cell response to urea. Am. J. Physiol. Renal Physiol. 278: F155–F164, 2000.—Growth factors and other stimuli increase the activity of phosphatidylinositol-3 kinase (PI3K), an SH2 domain-containing lipid kinase. In the murine kidney inner medullary mIMCD3 cell line, urea (200 mM) increased PI3K activity in a time-dependent fashion as measured by immune complex kinase assay. The PI3K effector, Akt, was also activated by urea as measured by anti-phospho-Akt immunoblotting. In addition, the Akt (and PI3K) effector, p70 S6 kinase, was activated by urea treatment in a PI3K-dependent fashion. PI3K inhibition potentiated the proapoptotic effect of hypertonic and urea stress. Urea treatment also induced the tyrosine phosphorylation of Shc and the recruitment to Shc of Grb2. Coexistence of activated Shc and PI3K in a macromolecular complex was suggested by the increase in PI3K activity evident in anti-Shc immunoprecipitates prepared from urea-treated cells. Taken together, these data suggest that PI3K may regulate physiological events in the renal medullary cell response to urea stress and that an upstream tyrosine kinase conferring activation of both PI3K and Shc may govern urea signaling in these cells.

METHODS

Cell culture and solute treatment. mIMCD3 cells were maintained and passaged as previously described (8). Prior to each experiment, cells were placed in serum-free medium for 24 h. Solute treatment consisted of the gentle, dropwise...
addition to ml MCD3 monolayers of an aliquot of concentrated urea (4.2 M) or NaCl (2.25 M) in sterile water, or an equal volume of NaCl (150 mM) in sterile water (sham treatment).

PI3K activity. Anti-PY immunoprecipitates (using antibody PY20) were assayed for PI3K activity as previously described (50, 58). Briefly, lipids and kinase reaction buffer (in final concentrations: ATP, 50 µM; MgCl 2, 5 mM; HEPES, 1 mM; 10% [γ-32P]ATP, 10 µCi) were added to the immunoprecipitates and incubated for 10 min at room temperature. Phosphatidylinositol (PI) was added to final concentration of 0.2 mg/ml. In experiments in which phosphatidylinositol 3,4,5-trisphosphate [PI(3,4,5)P 3] production was assayed, PI(4,5)P 2 and phosphatidylserine (in a 1:2 ratio) were added to 0.2 mg/ml final concentration of the combined lipids. Lipids were sonicated prior to use. Where indicated, immunoprecipitates were exposed to wortmannin (10 nM) for 10 min prior to the addition of lipid and kinase buffer. The lipid kinase assay was stopped by the addition of 80 µl of HCl (1 M) and 160 µl of methanol:chloroform (1:1 mixture). The lipid-containing organic phase was resolved on oxalate-coated thin-layer chromatography plates (Silica gel 60, MCB reagents; Merck, Rahway, NJ) developed in chloroform:methanol:water:ammonium hydroxide (60:40:11.3:2), or in n-propyl-2 M acetic acid (65:35) to quantify PI(3)P or PI(3,4,5)P 3 production, respectively. Radiolabeled spots corresponding to PI(3)P or PI(3,4,5)P 3 were quantified using a Bio-Rad Phosphoimager. In these and subsequent graphically depicted experiments, data from at least three separate experiments (each consisting of 2–4 replicates per experimental condition per experiment) were pooled, expressed as means ± SE, and then compared via t-test (Microsoft Excel software).

Immunoprecipitation and immunoblot analyses. For anti-Shc immunoprecipitates, ml MCD3 cell monolayers were lysed in situ with Shc lysis buffer (45) composed of (in mM) Tris hydroxide (0.42), 150 NaCl, 10 EDTA, 1 PMSF, 1 sodium orthovanadate, and 1 mM dithiobis (1,2-nitrobenzene disulfonic acid) (DTT), and then sub-

Electrophoresis, proteins were subjected to semi-dry transfer to polyvinylidene difluoride (PVDF) membranes, 0.1% SDS, 100 mM ampho-tin, and 1 mM leupeptin, and immunoprecipitated with monoclonal anti-Shc antibody (0.5 µg/ml; Transduction Laboratories) and Protein G beads (20 µl/sample; Pharmacia). Following extensive washing with both Shc lysis buffer and then Tris-buffered saline, immunoprecipitates were eluted from agarose beads by boiling in 1× Laemmlli sample buffer (43) prior to SDS-PAGE analysis. Alternatively, for p85 immunoblot analysis, anti-PY immunoprecipitates from RIPA-lysed cells were analyzed in similar fashion. Following electrophoresis, proteins were subjected to semi-dry transfer to polyvinylidene difluoride (PVDF) membrane and then incubated with anti-p85, anti-PY, anti-Grb2 (Santa Cruz Laboratories), or anti-phospho-Akt (New England Biolabs) primary antibodies and appropriate horseradish peroxidase-linked secondary antibodies (according to the manufacturer's directions). Blots were visualized with enhanced chemiluminescence (Renaissance; DuPont) followed by autoradiography.

Transfection and reporter gene assays. The construction of Egr-1-Luc, composed of 1.2 kb of the murine Egr-1 3′ flanking sequence (including the minimal promoter; Ref. 51) subcloned upstream of the promoterless luciferase reporter vector, pXP2 (37), has previously been described (12). BGT2X-Luc was prepared by subcloning a double-stranded oligonucleotide flanking the tandem repeats of the canine tonE enhancer element [TonE: Takenaka et al. (51), Miyakawa et al. (36)] upstream of the thymidine kinase promoter in BamHI/Hind III-eclased vector TK-Luc (37). (3′)Oligonucleotide sequence was gat ct act tgg tgg aaa agg cca gtc gat act tgg tgg aaa agg cca gca gat ctc tgg gac act tgg act tgg tgg aag cca ggt. (3′)Oligonucleotide sequence was agt ttt ctc tgg act ttt cca cca agt atc gaa.) Cells were transiently transfected with 10 µg Egr-1-Luc + 10 µg CMV-Gal per subconfluent 100-mm dish via electroporation as described (12). Luciferase and β-galactosidase activities in detergent lysates were determined as previously described (12); the former was normalized with respect to the latter (Luc/gal). Control and solute treatments were for 6 h (starting 48 h after transfection); inhibitors (wortmannin and LY-294002) were added 30 min prior to solute addition unless otherwise indicated.

p70 S6 kinase assay. Control- and solute-treated ml MCD3 monolayers were lysed with 1 ml of lysis buffer (20 mM Tris (pH 7.4), 1% Triton X-100, 10% glycerol, 137 mM NaCl, 2 mM EDTA, 25 mM β-glycerophosphate, 1 mM sodium orthovanadate, 1 mM PMSF, 10 µg/ml leupeptin; Ref. 17), then immunoprecipitated with anti-p70 S6 kinase COOH-terminal antibody (Santa Cruz Laboratories) for 60 min at 4°C. Immunoprecipitates were washed twice with lysis buffer, twice with kinase buffer (20 mM MOPS (pH 7.2), 25 mM β-glycerophosphate, 1.25 mM EGTA, 200 µM sodium ortho-

Valine 8

In situ with Shc lysis buffer (45) composed of (in mM) 30 Tris (pH 7.4); 150 NaCl, 10 EDTA, 1 PMSF, 1 sodium ortho-

Valine 8

Vanadium 8

Incubation was performed for 10 min at 30°C, after which the reactions were spotted on phosphocellulose paper, washed extensively with 0.75% phosphoric acid, and then washed with aceton prior to drying and scintillation counting. Kinase activity was expressed in raw counts per 100-mm dish. Data are presented as means ± SE of at least three separate experiments.

Cellular ATP content. An assay of intracellular ATP content was used as an index of cellular metabolic stress (29), as previously applied to renal cells (e.g., Ref. 48). This method was selected because other methods (e.g., propidium iodide exclusion and fluorescein diacetate conversion) were confounded by fluorescence of the vehicles and inhibitors used in the present study. ml MCD3 monolayers (in 24-well dishes and maintained in serum-free medium for 24 h) were treated for the indicated times with the indicated stressor for an additional 24 h. Cells were washed with ice-cold PBS and lysed in situ with 500 µl 0.5% Triton X-100. Plates were spun at 1,000 g for 10 min at 4°C. ATP content in 5 µl of lysate supernatant was quantitated using the ATP Determination Kit (Molecular Probes, Eugene, OR) by addition to 200 µl of complete assay buffer containing 1× reaction buffer [25 mM Tricine (pH 7.8), 5 mM MgSO 4, 0.1 mM EDTA, and 0.1 mM sodium azide], 1.5 mM DTT, and 1.875 µg/ml luciferase. Reaction was initiated by injection of 100 µl of luciferin stock (0.5 mM luciferin in 1× reaction buffer) in a Berthold Lumat LB-9501 luminometer. Data were normalized to protein content per well, as determined by the DC Protein Assay (Bio-Rad) according to the manufacturer's directions.

Apoptosis assays. Caspase-3 (casp32) microfluorescence assay was performed according to a modification of the method of Enari et al. (20). Briefly, cells were washed twice with ice-cold PBS, scraped into 50 µl of extraction buffer (50 mM Pipes-NaOH, pH 7.0, 50 mM KCl, 5 mM EGTA, 2 mM MgCl 2, 1 mM DTT, 20 µM cysteine, 1 mM PMSF, 1 µg/ml leupeptin, 1 µg/ml pepstatin A, 50 µg/ml antipain, and 10 µg/ml chymopapain) and lysed with five freeze-thaw cycles. Following centrifugation at 10,000 g for 12 min at 4°C, supernatants were assayed for protein concentration as above. Cell lysate (25 µg) was incubated in a reaction volume of 50 µl with fluorogenic substrate (N-acetyl-DEVD-MCA, 10 µM; BioMol), 100 mM HEPES-KOH, pH 7.5, 10% sucrose, 0.1% CHAPS, 10 mM DTT, and 0.1 mg/ml ovalbumin for 60 min at 30°C in a 96-well microtiter plate (Falcon). Enzyme activity
was detected by Cytofluor II (PerSeptive Biosystems, Framingham, MA) at an excitation wavelength of 360 nm and an emission wavelength of 460 nm. Annexin binding was determined using the ApoAlert Annexin V Apoptosis Kit (Clontech) in accordance with the manufacturer's directions. Cells were stains with propidium iodide and Annexin V-FITC as directed, then sorted (FL1 for Annexin V-FITC and FL2 for propidium iodide) on a Becton-Dickinson Calibur instrument, following gating upon a representative population of cells established by forward- and side-angle light scatter. Apoptosis (early) was ascribed to cells exhibiting Annexin staining >40 fluorescence units (FL1) and PI staining <300 fluorescence units (FL2).

RESULTS

Urea activates PI3K in a time-dependent fashion. In confluent, serum-deprived mIMCD3 cells, urea increased PI3K activity 3.2-fold [relative to basal (time 0)] at 1 min of treatment and 2.5-fold at 5 min of treatment (Fig. 1A). By 15 min, PI3K activity had returned to baseline. Sham treatment (treatment with isosmotic NaCl) exerted no effect upon PI3K activity at any time point examined (data not shown). In contrast to treatment with urea, NaCl treatment (200 mosmol/kgH2O) exhibited no effect at 1 min (Fig. 1A). PI3K activity was, however, increased by a factor of 3.1 at 5 min of NaCl treatment, and by a factor of 2.3 at 15 min of NaCl treatment. Although there was considerable variation among experiments in the magnitude of the PI3K activation by urea, the effect was observed in each of four separate experiments with peak activation ranging from 2.2- to 5.9-fold. To confirm that the immune complex kinase assay was indeed measuring PI3K activity and not that of a related lipid kinase, additional experiments were performed wherein PI(4,5)P2 was employed as the substrate. Results with PI(4,5)P2 precisely paralleled those observed using PI (data not shown). In addition, the effect of wortmannin, a pharmacological inhibitor of PI3K, was also examined. The PI3K activity in anti-PY immunoprecipitates from control and urea-treated cells was inhibited by ≥90% by wortmannin (10 nM) in vitro (data not shown). The effect of increasing concentrations of urea upon PI3K activity was next assessed. The 5 min rather than 1 min time point was selected for ease of reproducibility. The ability of urea to activate PI3K activity was not demonstrably dose-dependent within the range of 200–800 mM (Fig. 1B), although PI3K activity was increased relative to control at each of these concentrations.

In correlative fashion, the effect of solute treatment upon abundance of the SH2 domain-containing PI3K subunit, p85, in the anti-PY immunoprecipitates used for measuring PI3K activity was determined via immunoblot analysis. Consistent with the data presented in Fig. 1A, the abundance of p85 in anti-PY immunoprecipitates (Fig. 2) was maximal following 1 min of urea treatment and was at least twofold greater than at time 0. Thus, the urea-promoted increase in PI3K activity (Fig. 1A) was due to an increase in the association of PI3K with anti-PY immunoprecipitates. In similar fashion, p85 abundance in the anti-PY immunoprecipitates from NaCl-treated cells was increased at the 5- and 15-min time points, but not at 1 min of treatment (Fig. 2).

Effectors of PI3K in urea stress. Urea activates transcription of the immediate-early gene, Egr-1 (8), through
a signaling pathway bearing hallmarks of a PKC- (11) and Ras-mediated event. Because Ras represents a potential upstream activator of both PI3K activation and Egr-1 transcription, we hypothesized that PI3K activation may play a role in urea-inducible Egr-1 transcription. mIMCD3 cells were transiently transfected with a luciferase reporter construct driven by 1.2 kb of the murine Egr-1 proximal 5' flanking sequence (diagrammed in Fig. 3A). As previously reported (12), urea (200 mM) increased Egr-1 transcription in luciferase reporter gene assay by sevenfold (Fig. 3A). The pharmacological inhibitor of PI3K action, wortmannin (10 nM), failed to inhibit urea-inducible transcription (Fig. 3A). Higher concentrations of wortmannin (100 nM and 1 µM) and another PI3K inhibitor, LY-294002 (10 and 30 µM), also failed to inhibit this effect (data not shown). Because of the ability of hypertonic stress to activate PI3K, albeit at later time points than urea, and the incompletely understood nature of hypertonicity-inducible gene regulation, the role of PI3K activation in a model of hypertonicity-inducible gene transcription was examined in parallel. Tandem repeats of the tonicity-responsive element (TonE) of the betaine transporter gene (BGT1; Ref. 51) were subcloned upstream of the thymidine kinase (TK) promoter and luciferase reporter gene (Fig. 3B). Consistent with the observations of others (36, 51), hypertonic NaCl (200 mosmol/kgH2O) increased reporter gene activity 10-fold. Pretreatment with wortmannin (10 nM to 1 µM) failed to abrogate NaCl-inducible transcription.

p70 S6 kinase is a potential physiological effector of PI3K (53). Urea (200 mM) significantly increased p70 S6 kinase activity in a time-dependent fashion (Fig. 4A); the effect was detectable as early as 1 min of treatment (2-fold increase) and remained evident at 15 min of treatment (3-fold increase). To bolster the argument that urea-inducible p70 S6 kinase activity was a consequence of urea-inducible PI3K activity, the effect of wortmannin and LY-294002 pretreatment upon urea-inducible p70 S6 kinase activity was examined. At 5 min of treatment, urea increased p70 S6 kinase activity by 75% (Fig. 4B). Pretreatment with either wortmannin (10 nM) or LY-294002 (10 µM) exerted no statistically significant effect upon basal p70 S6 kinase activity but significantly inhibited the urea-inducible increment in S6 kinase activity by 55% and 100%, respectively.

Recent data suggest that the protein kinase Akt mediates the effects of PI3K upon p70 S6 kinase activation (reviewed in Ref. 21). Urea treatment (200 mM) increased phosphorylation of Akt as detected by anti-phospho-Akt immunoblotting (Fig. 5A). The effect of NaCl was consistently less pronounced. Both wortmannin (10 and 100 nM) and LY-294002 (10 and 30 µM) inhibited the effect of urea upon Akt phosphorylation in a dose-dependent fashion (Fig. 5B), suggesting a role for PI3K activation in urea-inducible Akt activation. The ability of both urea and NaCl to increase Akt phosphorylation was steeply dose-dependent (Fig. 6), at the time points correlated with maximal induction (5 min for urea and 15 min for NaCl). At 100 and 200 mosmol/kgH2O solute, the urea effect exceeded that of NaCl.

We have previously shown that urea treatment results in the activation of the SH2 domain-containing phospholipase, PLC-γ. We hypothesized that a urea-activable (tyrosine-phosphorylated) upstream receptor or non-receptor tyrosine kinase recruited and activated PLC-γ. The present data imply activation of another tyrosine kinase effector, PI3K. To further implicate an upstream activating kinase, the ability of urea to activate (induce the tyrosine phosphorylation of) the RTK effector and adapter molecule, Shc, was examined. Under control (sham-treated) conditions, immunoprecipitated Shc exhibited demonstrable tyrosine phosphorylation by anti-PY immunoblotting (Fig. 7; left). Follow-
ing urea treatment, but not NaCl treatment, the degree of tyrosine phosphorylation was markedly upregulated. In addition, the abundance of the adapter molecule and Shc interaction partner, Grb2, was also markedly increased in anti-Shc immunoprecipitates from urea-treated cells (Fig. 7, right). The ability of urea to activate PI3K and Shc and to recruit Grb2 strongly suggested the presence of a phosphotyrosine-bearing upstream activator. It was hypothesized that such an activator, as has been observed in other models, would serve as a molecular "scaffold" for recruitment of multiple effectors. Therefore, the ability of anti-Shc immunoprecipitates from urea-treated cells to exhibit increased PI3K activity (diagrammed in Fig. 8A) was examined. Consistent with this model, urea treatment of cells reproducibly increased PI3K activity in anti-Shc immunoprecipitates (Fig. 8B).

Physiological consequences of PI3K inhibition. To determine the possible contributions of PI3K action to a physiological consequence of hypertonic stress and elevated urea concentration, mIMCD3 cells were treated with PI3K inhibitors (LY-294002, 30 µM; or wortmannin, 100 nM) and evaluated with respect to intracellular ATP content as an index of cellular metabolic stress (29), as previously applied to renal cells (e.g., Ref. 48). The effects of the two inhibitors were comparable. Pretreatment with LY-294002 did not influence relative ATP content (normalized to cell protein) under basal conditions in mIMCD3 cells [n = 3 separate experiments, with 3–6 individual determinations (replicates) per experimental condition; data not shown]. Urea decreased cell ATP content by only 5% in the absence of PI3K inhibition but by 18% in the presence of the inhibitor (P < 0.05; LY-294002 vs. vehicle). Similarly, NaCl decreased cellular ATP content by 18% in the absence of PI3K inhibition and by 29% in its presence (P < 0.05; LY-294002 vs. +LY-294002). These data suggested that whereas PI3K inhibition in isolation produced no adverse effect, its superimposition upon osmotic and urea stress exacerbated an index of metabolic stress.

Because others have shown that hyperosmotic solutes, including NaCl and urea (44), may induce apoptosis and because PI3K activation has been implicated in

Fig. 4. p70 S6 kinase activity is increased by urea in a PI3K-dependent fashion. A: effect of urea (200 mM) upon p70 S6 kinase activity (expressed relative to time 0) was measured by immune complex kinase assay and expressed as a function of time of treatment (in minutes). Data from at least three separate experiments (each with determinations performed in duplicate) are depicted as means ± SE. Not shown, sham treatment exerted no effect upon p70 S6 kinase activity. *Statistical significance (P < 0.05) with respect to time 0. B: effect of control and urea (+Urea; 200 mM) treatment upon p70 S6 kinase activity was measured by immune complex kinase assay and expressed relative to control, in absence of pretreatment (open bars), and following pretreatment with wortmannin (10 nM for 30 min; light gray bars) or LY-294002 (10 µM for 30 min; solid black bars). Data are expressed relative to control and depict means ± SE of at least 3–4 separate experiments with determinations performed in duplicate. *P < 0.05 and †P < 0.05, statistically significant with respect to vehicle-treated control and vehicle + urea treatment, respectively.

Fig. 5. Urea induces Akt phosphorylation in a PI3K-dependent fashion. Anti-phospho-Akt immunoblot of detergent lysates prepared from mIMCD3 cells treated with urea and NaCl at indicated concentrations for the indicated intervals (A) or with control (−) or urea (+; 200 mM for 5 min) in absence (−Vehicle) or presence of 30-min pretreatment with indicated concentration of wortmannin (WT) or LY-294002 (LY) (B).
this process, a possible protective role of PI3K signaling was evaluated in this context. Specifically, the ability of PI3K inhibition by wortmannin and LY-294002 to potentiate urea- and NaCl-inducible apoptosis was investigated. Caspase-3 activation has been described as a “rubicon” of apoptosis, and correlates closely with this phenomenon in vitro and in vivo (56). Wortmannin treatment increased caspase-3 activity in mIMCD3 cells by 68% in the absence of hypertonic or urea stress (Fig. 9); however, this was not significantly different from treatment with vehicle alone. Urea (200 mM) exerted no adverse effect upon caspase activity, consistent with prior observations concerning the ability of the mIMCD3 cell line to tolerate this solute; however, a modest (30%) protective effect could not be excluded. In the presence of wortmannin pretreatment, urea-associated apoptosis was increased 171%. Urea at 400 mM and NaCl at 200 mosmol/kg H2O (100 mM) substantially increased caspase-3 activity. The effect of these solutes was increased by 266% and 185%, respectively, in the presence of wortmannin pretreatment. Similar findings were observed when cells were pretreated with the phospholipase A2 inhibitor, quinacrine, or the PLC inhibitor, PLC-g, receptor tyrosine kinase-specific phospholipase C isoform. B: relative PI3K activity in anti-Shc immunoprecipitates prepared from cells receiving control treatment or treatment with urea (200 mM for 5 min). *P < 0.05 with respect to control treatment.
U-73122, there was only a modest effect (≤40% increase) upon NaCl- and urea-inducible apoptosis (as measured via caspase activation), which was indistinguishable from the effect of these inhibitors upon control-treated cells (data not shown).

To further corroborate these findings, the effect of PI3K inhibition upon urea and osmotic tolerance was examined using another index of apoptosis. Initiation of apoptosis is followed by translocation of phosphatidylinerine from the inner to the outer leaflet of the cell membrane, a phenomenon detectable by cell staining with fluorophore-conjugated annexin V. Following gating upon a homogeneous population of FACS-sorted cells with respect to forward- and side-angle light scatter (see METHODS), apoptosis (early) was assigned to cells exhibiting PI staining <30 U and annexin staining >40 U. Under control conditions, wortmannin pretreatment increased the percentage of annexin V-positive cells by only 44.6% (Table 1). In the presence of urea (400 mM) or NaCl (200 mM), in contrast, wortmannin pretreatment increased the percentage of annexin V-positive (apoptotic) cells by 178% and 91%, respectively.

**DISCUSSION**

These data indicate that urea, in concentrations physiologically relevant to the renal medulla in vivo, activated the lipid kinase and RTK effector, PI3K, as well as the potential physiological PI3K effectors, Akt and p70 S6 kinase. Inhibition of this pathway appeared to modestly impair urea tolerance and enhance urea- and hypertonicity-associated apoptosis. Urea also induced tyrosine phosphorylation of Shc and recruitment to Shc of Grb2. Furthermore, PI3K activity was increased in anti-Shc immunoprecipitates prepared from urea-treated cells. In aggregate, these findings appear to support the previously proposed model of receptor or non-receptor tyrosine kinase in the initiation of urea signaling in cells of the renal medulla (11). Activation of membrane-associated kinases has been described in hypertonic signaling in yeast (33, 34) and recently in higher eukaryotes (41) but not in the setting of elevated urea concentrations. In light of accumulating evidence including urea-inducible immediate-early gene transcription (8), ERK and Elk-1 activation (7), IP3 generation (11), and activation of PLC-γ (11), the involvement of an RTK-mediated signaling pathway appears increasingly likely in urea stress.

To our knowledge, these are the first studies to directly measure PI3K activity in response to hypertonic or urea stress and to implicate this pathway in osmotic and urea tolerance. Activation of PI3K has previously been suggested in other related contexts through the use of inhibitor and dominant negative-acting expression constructs. Activation of PI3K in response to elevated glucose concentrations in hepatocytes has been inferred through the use of dominant negative PI3K subunits (1). Inhibitor studies have implicated a role for PI3K in the hyperosmotic shrinkage-induced Na⁺-dependent glutamine uptake in muscle cells (32). Cell swelling in the setting of hypotonic stress has been associated with PI3K activation in one nonrenal model (28). However, in marked contrast to urea stress (7) and hypotonicity in renal (61) and other cells (42, 46, 47), swelling in this hepatic model was not associated with ERK activation (28). In the C6 glioma cell line, PI3K inhibitors failed to influence hypotonicity-inducible ERK activation (49). In addition to IP3, as discussed above, and PI(3,4,5)P3 (the lipid product of PI3K action), the role of another membrane-derived phospholipid has recently been described in osmotic stress signaling. PI(3,5)P2 is produced upon osmotic shock of yeast, presumably via a PI5K activity (18). Interestingly, osmotic stress in mammalian cells decreases abundance of this lipid (18).

Activations of PI3K and of Akt have been implicated in the prevention of apoptosis (21), and apoptosis has previously been demonstrated in renal medullary cells in response to both elevated urea concentrations and hypertonicity (44). In the present study, pharmacological inhibition of PI3K action potentiated the proapop-

**Table 1.** PI3K inhibition potentiates urea- and hypertonicity-inducible annexin V binding

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<th>Annexin V Positivity, %</th>
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Values are means ± SE. Effect of wortmannin pretreatment (100 nM for 30 min) upon apoptosis as measured by percentage of cells exhibiting FITC-annexin V binding positivity (>40 fluorescence units; see METHODS) in control-treated mIMCD3 cells and in cells treated with urea (400 mM for 4 h) or NaCl (200 mM for 4 h). Data are derived from the counting of 10,000 cells per experimental condition as described (see METHODS); the depicted experiment is representative of 2 such experiments. PI3K, phosphatidylinositol-3-kinase. *Percent increase in FITC-annexin V-positive cells associated with wortmannin treatment, relative to control treatment (−Wortmannin).
totic effect of urea and hypertonicity as determined by both a sensitive assay of caspase-3 activation and by an annexin V binding assay. It is therefore likely that PI3K action is essential for maximal osmotic and urea tolerance in these cells. This possibility is underscored by the decrement in cell ATP content associated with PI3K inhibition in the presence of hypertonic or urea treatment but not control treatment. The possible involvement of a wortmannin- and LY-294002-sensitive non-PI3K signaling intermediate cannot be excluded at present. The precise nature of this potential PI3K-associated protective (anti-apoptotic) pathway in the present context remains speculative. Such a protective effect would be of limited significance at solute concentrations not associated with a proapoptotic effect in this model (e.g., urea 200 mM). Pronounced activation of PI3K (Fig. 1) and PI3K effectors (e.g., Fig. 6), however, is observed under such conditions, raising the possibility of an additional role for PI3K-mediated signaling in the medullary cell response to urea and/or hypertonicity.

A second potential function of PI3K concerns mitogenic signaling; however, this would appear unlikely in the present model. Although urea activates DNA synthesis in canine renal MDCK and porcine renal LLC-PK₁ cells without increasing cell number (10), no such effect is evident in mIMCD3 cells (8). A role for PI3K in regulating cytoskeletal dynamics and cell adhesion properties has been demonstrated in other models; a similar function can be envisioned in the context of urea (or hypertonic) stress. Hypertonic stress activated p125Fak, a component of focal adhesion complexes (59), and PI3K activation was required for p125Fak phosphorylation in the platelet-derived growth factor response (40). Hypertonic stress also influences actin expression and polymerization (3, 24, 52); the effect of urea upon these phenomena has not been examined. Interestingly, members of the Rho family of small GTP-binding proteins (including Rho, Rac, and Cdc42), which regulate focal adhesion formation and actin cytoskeletal rearrangement (39), also regulate activation of the toxicity (NaCl)-responsive MAPK, SAPK/JNK (14, 35, 38).

Urea-inducible activation of p70 S6 kinase appears to be a consequence of PI3K activation. Potential effectors of p70 S6 kinase are few; until recently, the only known physiological substrate of p70 S6 kinase was the S6 protein of the ribosomal 40S subunit. Phosphorylation of S6 is essential for cell proliferation and for the increment in protein synthesis that accompanies the G0/G1 cell cycle transition (19, 27). Interestingly, NaCl actually inhibits protein synthesis (e.g., Ref. 13, and references therein) but urea fails to do so (9). In addition, inhibitors of protein synthesis themselves may activate p70 S6 kinase (4), suggesting that the NaCl (but not the urea) effect may be a consequence of this phenomenon. p70 S6 kinase may also activate the nuclear protein and CAMP-dependent protein kinase A effector, CAMP-responsive element mediator (CREM) (15, 16). Urea, however, failed to activate transcription through tandem copies of the CAMP-responsive element in reporter gene experiments (Zhang and Cohen, unpublished observation).

The ability of urea to activate PLC-γ (11), PI3K, and Shc and the association of PI3K activity with anti-Shc immunoprecipitates suggests the presence of a urea-activable upstream receptor or non-receptor tyrosine kinase. In model systems unrelated to the kidney medulla, severe hypertonic stress induced the activation of the receptors for epidermal growth factor (EGF), tumor necrosis growth factor (TNF), and interleukin-1 (IL-1) (41), a process potentially related to an inhibition in receptor-directed phosphatase activity (26). Indiscriminate activation of membrane-associated RTKs (e.g., EGF receptor (EGFR), hepatocyte growth factor receptor (HGF), etc.) in contrast, is not observed in the present model (data not shown). Accumulating data indicate that signaling events engendered by urea treatment, despite some overlap, are largely dissimilar from those activated by hypertonicity with respect to MAPK isofoms activated and, ultimately, genes expressed (reviewed in Refs. 30 and 31). In addition, whereas the response to hypertonicity appears to be universal among cell lines examined, the response to urea has been restricted to a small subset of renal epithelial cells including MDCK and mIMCD3 cells. Nonetheless, in a fashion potentially similar to that of hypertonicity, the membrane-permeant solute urea likely influences activation of an upstream receptor or non-receptor tyrosine kinase, the identity of which remains obscure.

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