Ras signaling in the inner medullary cell response to urea and NaCl

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Tian, Wei, Gerry R. Ross, and David M. Cohen. Ras signaling in the inner medullary cell response to urea and NaCl. Am. J. Physiol. Cell Physiol. 278: C372–C380, 2000.—The small guanine nucleotide-binding protein Ras, activated by peptide mitogens and other stimuli, regulates downstream signaling events to influence transcription. The role of Ras in solute signaling to gene regulation was investigated in the murine inner medullary collecting duct (mIMCD3) cell line. Urea treatment (100–200 mM), but not sham treatment, increased Ras activation 124% at 2 min; the effect of NaCl did not achieve statistical significance. To determine the contribution of Ras activation to urea-inducible signal transduction, mIMCD3 cells were stably transfected with an expression plasmid encoding a dominant negative-acting N17Ras mutant driven by a dexamethasone-inducible (murine mammary tumor virus) promoter. After 24 h of induction, selected cell lines exhibited sufficient N17Ras overexpression to abolish epidermal growth factor- and hypotonicity-mediated signaling to extracellular signal-regulated kinase (ERK) phosphorylation, as determined by immunoblotting. Conditional N17Ras overexpression inhibited urea- and NaCl-inducible ERK phosphorylation by 40–50%, but only at 15 min, and not 5 min, of treatment. N17Ras induction, however, almost completely inhibited urea-inducible Egr-1 transcription, as quantitated by luciferase reporter gene assay, but failed to influence tonicity-inducible (TonE-mediated) transcription. N17Ras overexpression also blocked urea-inducible expression of the transcription factor Gadd153 but did not influence osmotic or urea-inducible apoptosis. In addition, urea treatment induced recruitment of the Ras activator Sos to the plasma membrane. Taken together, these observations suggest a role for Ras signaling in the IMCD cell response to urea stress.

hypertonicity; cell volume regulation, Gadd153; extracellular signal-regulated kinase; mitogen-activated protein kinase

METHODS

Cell culture and solute treatment. mIMCD3 cells (American Type Culture Collection) were maintained and passaged as
previously described (7). 3T3 cells were obtained from the Vollum Institute for Advanced Biomedical Research at Oregon Health Sciences University and were maintained in DMEM-F-12 (J RH, Lenexa, KS) supplemented with 10% fetal bovine serum (J RH). Before each experiment, cells were placed in serum-free medium for 24 h. Solute treatment consisted of 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).

Nonisotopic Ras GTP loading assay. Ras activation is defined as the percentage of Ras molecules in the active GTP-bound state, i.e., Ras-bound GTP/(Ras-bound GDP + Ras-bound GDP) × 100. and was measured using a recent modification (28) of our original protocol (27). Briefly, cells were lysed in HEPES-based buffer containing 1% Nonidet P-40, and protein G-agarose beads preincubated with the rat monoclonal pan-Ras antibody Y13-259 (Zymed) and a rabbit anti-rat IgG-Fc secondary antibody (experimental sample) were added to half of the lysate. Beads preincubated with (nonspecific) rat IgGs and the rabbit anti-rat secondary antibody (control sample) were added to the other half of the sample. Samples were shaken for 1 h, which quantitatively immunoprecipitates Ras; MgCl₂ and high salt concentration in the buffer inhibit Ras-directed GAP activity and GTP/GDP dissociation from Ras. In addition, Y13-259 is a Ras-neutralizing antibody that inhibits interaction of GEF and GAP with Ras. GDP and GTP were quantitatively eluted from the immunoprecipitates by heating, a process that destroys <5% of these nucleotides. GTP was measured after conversion to ATP with use of the enzyme nucleoside diphosphate kinase. ATP was then measured by the luciferase-luciferin system (28). GDP was first converted to GTP by GTPase activity of pyruvate kinase and phosphoenolpyruvate and was then quantitated as described above. Standard curves were prepared with each assay, and the amount of sample GDP and GTP was calculated as the difference between the experimental and control samples. Because the GDP assay measures GTP + GDP, the amount of GTP is subtracted from the sum to yield the amount of GDP. Both assays are sensitive to 1 fmol of nucleotide (28).

Immunoblot analysis. Detergent lysates were prepared from serum-deprived ml MCD3 cells, as previously described (7). Equal amounts of protein (µg) were loaded per lane and subjected to SDS-PAGE. After electrophoresis, proteins were subjected to semidry transfer to polyvinylidine difluoride membrane and then incubated with anti-Gadd153, anti-Ras, or anti-Sos (all from Santa Cruz Laboratories) or anti-P-ERK, anti-P-Stress-activated protein kinase (SAPK), anti-P-p38, 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 (Renais- sance, DuPont) and then by fluorography. For membrane protein preparation, monolayers were washed twice with ice-cold PBS, scrapped into 600 µl of lysis buffer (20 mM Tris (pH 7.5), 2 mM EDTA, 2 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol (DTT), 20 µg/ml leupeptin, and 80 µg/ml aprotinin), and subjected to homogenization (30 strokes in a Dounce homogenizer) with a tight pestle. Lysates were centrifuged at 130,000 g for 60 min at 4°C. The resultant supernatant was retained as cytoplasmic lysate. The pellet, representing the membrane fraction, was resuspended by homogenization (Dounce homogenizer) with 300 µl of lysis buffer supplemented with 1.2% Triton X-100 and then clarified in a microcentrifuge at 12,000 rpm for 5 min at 4°C. Protein concentration in whole cell lysates and subcellular fractions was quantitated with the Bio-Rad protein assay kit according to the method of Bradford (2).

Transfection and reporter gene assays. The construction of Egr-1-Luc, comprised of 1.2 kb of the murine Egr-1 5'-flanking sequence (including the minimal promoter) (31) and subcloned upstream of the promoterless luciferase reporter vector pXP2 (24), has previously been described (10); BGT-2X-Luc was prepared by subcloning a double-stranded oligonucleotide encoding two tandem repeats of the c-myc oncogene enhancer element (TonE) (23, 29) upstream of the thymidine kinase promoter in BamHI/HindIII-deaaved vector TK-Luc (24). The 5'-oligonucleotide sequence was GAT CCT ACT TGG TGG AAAAGT CCA GTG GAC ATG; TGG AAAAGT CCA GA; the 3'-oligonucleotide sequence was AGC TTC TGG TGG AAAAGT CCA AAAGT CCA GAAGT. Cells were transiently transfected with 10 µg of Egr-1-Luc + 3 µg of cytomegalovirus-Gal per subconfluent 100-mm dish via electroporation, as described elsewhere (10). pMMTV-Ras-Asn17 (N17Ras) expression vector was provided by G. Cooper (5). With use of this vector, ml MCD3 cells were stably transfected via electroporation (<1% transfection efficiency) and selected in G418-containing medium commencing 48 h after transfection. When individual colonies became visible (i.e., >1 mm diameter), culture medium was removed and cells were aspirated with several microliters of medium with use of a 200-µl tip on a Gilson-style pipettor. Cells obtained from individual colonies were dispersed into labeled wells of 24-well plates prefilled with prewarmed DMEM-F-12 + 10% fetal bovine serum. Wells achieved confluence in ~2 wk and were propagated for further study. Luciferase and β-galactosidase activities in detergent lysates were determined as previously described (10); the former was normalized with respect to the latter. The duration of control and solute treatments was 6 h (starting 48 h after transfection). Dexa- methasone treatment for 24 h decreased total protein content in cell monolayers by ~5% in untransfected cells and by ~14% in N17Ras-B7 cells.

Caspase-3 assay. Caspase-3 (cpp32) microfluorescence assay was performed according to a modification of the method of Enari et al. (12). 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 MgCl₂, 1 mM DTT, 20 µM cytochalasin B, 1 mM phenylmethylsulfonyl fluoride, 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. After centrifugation at 10,000 g for 12 min at 4°C, supernatants were assayed for protein concentration, as described above. Cell lysate (25 µg) was incubated in a reaction volume of 50 µl with fluorescent substrate (N-acetyl-DEVD-MCA, 10 µM; BioMol), 100 mM HEPES-KOH, pH 7.5, 10% sucrose, 0.1% [3-(cholamidopropyl)dimethylammonio]-1-propanesulfonate, 1 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.

RESULTS

Urea increases Ras activation in ml MCD3 cells. The ability of urea and NaCl to activate Ras was investigated using an enzymatic method to measure Ras-GTP/GDP (27, 28). Under basal conditions, ~5% of immunoprecipitable Ras was activated (GTP bound; Fig. 1). After 2 min of urea treatment (200 mM or 200 mosmol/kg H₂O), Ras activation was increased 124% (to 15.3%) and decreased promptly thereafter. At 200 mosmol/
promoter (kindly provided by G. Cooper) (5). Of responsive murine mammary tumor virus (MMTV) under the control of the inducible dexamethasone-dominant negative mutant (N17) Ras isoform stably transfected with an expression vector encoding a urea-stressed cell was examined. mIMCD3 cells were the role of this signaling event in the phenotype of the acting Ras isoform N17Ras.

Nonrenal 3T3 cell line, urea exerted no effect on Ras however, did not achieve statistical significance. In the 44% at 5 and 10 min, respectively. The effect of NaCl, kgH2O, NaCl increased Ras activation by only 38 and 44% at 5 and 10 min, respectively. The effect of NaCl, however, did not achieve statistical significance. In the nonrenal 3T3 cell line, urea exerted no effect on Ras activation (data not shown).

Generation of mIMCD3 cell lines exhibiting high-level inducible expression of the dominant negative-acting Ras isoform N17Ras. Because of these biochemical data demonstrating urea-inducible Ras activation, the role of this signaling event in the phenotype of the urea-stressed cell was examined. mIMCD3 cells were stably transfected with an expression vector encoding a dominant negative-acting mutant (N17) Ras isoform under the control of the inducible dexamethasone-responsive murine mammary tumor virus (MMTV) promoter (kindly provided by G. Cooper) (5). Of 50 clones isolated after stable transfection and antibiotic selection, several exhibited marked induction of the mutant Ras in response to dexamethasone treatment (300 nM for 24 h). Clone B7 (lane 7, Fig. 2) was selected for further study because of its relatively low level of basal Ras expression and its marked induction in response to dexamethasone treatment. To confirm that overexpression of the inducible N17Ras-encoding plasmid permitted inducible inhibition of Ras-dependent events, control experiments were performed. Although there are no documented Ras-dependent events in this cell line, the effects of hypotonicity and the peptide mitogen epidermal growth factor on ERK activation (phosphorylation) have been shown to be Ras dependent in other models (e.g., Ref. 32). In untransfected mIMCD3 cells, both of these stimuli, as well as phorbol ester (12-O-tetradecanoylphorbol-13-acetate) treatment, markedly increased ERK phosphorylation, as determined by anti-P-ERK immunoblotting (Fig. 3). Under

none of these conditions, however, was ERK activation substantially inhibited by dexamethasone pretreatment. In contrast, in the N17Ras-B7 cell line, ERK phosphorylation in response to hypotonic stress and treatment with epidermal growth factor and 12-O-tetradecanoylphorbol-13-acetate was markedly attenuated by dexamethasone pretreatment, implying a role for Ras in these activation events. These experiments appeared to validate the present model for investigating the contribution of Ras signaling to the urea-stressed phenotype.

Effect of N17Ras overexpression on urea and NaCl signaling to ERK activation. In untransfected cells, urea and NaCl increased ERK phosphorylation at 5 and 15 min of treatment at 200 and 800 mosmol/kgH2O solute, consistent with our prior observations and observations of others (1, 35, 36) (Fig. 4). In parental mIMCD3 cells, dexamethasone exerted no effect on ERK phosphorylation under any experimental condition examined, nor did it have an effect on the level of (endogenous native) Ras expression. In the N17Ras-B7 cells, dexamethasone treatment produced a pronounced increase in Ras immunoreactivity, as demonstrated by immunoblotting. At lower concentrations of urea (200 mM), the effect of dexamethasone treatment (i.e., N17Ras induction) relative to no treatment was demon-
strable only at 15 min. At 5 min of treatment, the effect did not achieve statistical significance (Fig. 5). In the absence of dexamethasone treatment, the effect of 200 mosmol/kgH2O urea and NaCl in the N17Ras-B7 cell line was substantially less than that of untransfected cells, suggesting a small amount of basal (constitutive) expression of N17Ras. At high concentrations of solute (800 mosM), there was no consistent effect of N17Ras overexpression on urea- or NaCl-inducible ERK phosphorylation. Data from three separate experiments are combined in Fig. 5. At 15 min of solute treatment in N17Ras-B7 cells, dexamethasone treatment inhibited urea- and NaCl-inducible ERK phosphorylation by 38 and 46%, respectively, whereas at 5 min of treatment, there was no effect. Importantly, these results were corroborated in a second N17Ras-overexpressing cell line, N17Ras-B9 (Fig. 2; data not shown).

Effect of N17Ras overexpression on urea and NaCl signaling to other MAPKs. In light of the findings described above and the ability of urea and NaCl to activate other members of the MAPK family, the effect of dominant negative Ras expression on solute-inducible p38 and SAPK activation was investigated. NaCl (400 mosM for 15 min) markedly activated p38 and hypotonicity (50% medium dilution for 5 min) minimally activated p38, consistent with prior observations of the authors and others, whereas urea had no discernible effect (Fig. 6). Dexamethasone pretreatment had no effect on either of these signaling events, implying specificity of the ERK events. With respect to SAPK,
hypertonicity, but not urea or hypotonicity, induced activation (phosphorylation). Similar to the case with p38, N17Ras overexpression failed to inhibit the effect, a finding previously noted by Kawasaki et al. (18).

Overexpression of N17Ras abrogates transcriptional regulation by urea but not by NaCl. Because immediate-early gene transcription in response to urea treatment is mediated via serum response element (SRE)/Ets motifs and because this process is likely Ras dependent (4), the ability of dominant negative-acting N17Ras to block the effect of urea on transcription was examined. When N17Ras-B7 cells were transiently transfected with a luciferase reporter gene driven by the proximal 1.2 kb of the murine Egr-1 5'-flanking sequence, urea increased reporter gene activity ~19-fold, consistent with earlier observations (10) (Fig. 7). Pretreatment with dexamethasone inhibited basal Egr-1 transcription by only 22%, whereas it inhibited urea-inducible transcription by fully 70%. Importantly, in untransfected cells, the effect of dexamethasone pretreatment on urea-inducible Egr-1 transcription was negligible (data not shown). For comparison purposes, the effect of dominant negative N17Ras overexpression on NaCl-inducible transcription mediated via the osmotic response element (ORE) (15)/TonE (29) was examined in parallel. The BGT1 TonE is a well-characterized toxicity-responsive cis-acting element; two tandem repeats of this element were subcloned upstream of the thymidine kinase promoter in a luciferase reporter vector. Consistent with others' observations (29), hypertonicity increased reporter gene activity by 21-fold; however, dexamethasone pretreatment failed to influence this effect. Urea (200 mM) fails to appreciably activate TonE-mediated transcription (data not shown), and NaCl (200 mosmol/kgH2O) fails to exert a substantial effect on Egr-1 transcription (10); therefore, the effect of N17Ras expression on these events was not examined. Overexpression of N17Ras blocks urea-inducible expression of Gadd153. Stressors of the endoplasmic reticulum induce expression of the transcription factor Gadd153. Although not a bona fide endoplasmic reticulum stressor, urea increases expression of Gadd153 at the protein and mRNA levels (37). As an additional manifestation of the urea-stressed phenotype, the effect of Ras inhibition on Gadd153 expression was examined. In control (untransfected) cells (Fig. 8), urea treatment (200 mM for 6 h) increased Gadd153 protein expression by immunoblotting, as did the potent positive controls (34) tunicamycin and cadmium chloride. Dexamethasone pretreatment exerted no effect on the ability of any of these stressors to activate Gadd153 expression. In the N17Ras-B7 cells, all three stimuli increased Gadd153 protein abundance relative to control; however, the effect of cadmium chloride was much more pronounced than in untransfected cells. Importantly, N17Ras overexpression virtually abolished the effect of urea on Gadd153 immunoreactivity and sub-
stantially inhibited the effects of tunicamycin and cadmium chloride.

Overexpression of N17Ras does not influence solute-inducible apoptosis. It has previously been shown that urea and NaCl may induce apoptosis in mIMCD3 cells (26) and that interruption of urea- and NaCl-inducible signaling events may markedly exacerbate this phenomenon (38). Therefore, the effect of Ras inhibition on solute-inducible apoptosis was examined using the sensitive cpp32 (caspase-3) assay (12, 38). In the present model, 400 mM urea modestly increased apoptosis whereas 200 mosM NaCl markedly increased apoptosis (Fig. 9). (Urea at 200 mM, used in the present and previous signaling studies, fails to increase caspase-3 activity.) Pretreatment with dexamethasone to induce N17Ras expression neither inhibited nor potentiated these effects. In addition, urea and NaCl induce phosphorylation of Akt (38), an event correlated with apoptosis. Inducible overexpression of N17Ras had no effect on urea- or NaCl-inducible Akt phosphorylation, as determined by anti-P-Akt immunoblotting (data not shown).

Solute-inducible Ras activation may be Sos mediated. The guanine nucleotide exchange factor Sos mediates Ras activation in response to mitogens in multiple cell culture models. Because Sos activation of Ras requires membrane targeting, the ability of urea to induce translocation of Sos to the plasma membrane was assessed through immunoblot analysis of membrane preparations. Urea increased Sos abundance in the plasma membrane by ~100% (Fig. 10).

**DISCUSSION**

In this series of experiments it is shown that 1) urea activates Ras, 2) inducible overexpression of dominant negative-acting N17Ras inhibits urea-inducible ERK activation in a sharply time-dependent fashion, 3) dominant negative Ras markedly inhibits urea-inducible Egr-1 transcription without affecting hypertonicity-inducible transcription, 4) dominant negative Ras blocks urea-inducible Gadd153 expression, and 5) the ability of urea to activate Ras may be mediated through the GEF Sos.

Although the ability of urea to activate Ras has not previously been described in any context, Ras activation has been inferred in other anisotonic contexts. Although Ras activation was not directly observed, hypotonicity-inducible ERK activation was inhibitable by N17Ras expression in a human intestinal cell line (32). In similar fashion, Ras activation in response to hypertonic stress has been implicated through biochemical effects of overexpressing dominant negative-acting Ras isoforms (18). Terada et al. (30) demonstrated activation of the Ras effector Raf in hypertonicity-stressed Madin-Darby canine kidney cells. In aggregate, these data suggest a possible role for Ras activation in cell volume regulation, as was postulated by Lang et al. (22), in part on the basis of the perturbed volume set point and regulatory responses observed in cells stably transfected with a constitutively active (oncogenic) Ha-Ras isoform. The inability of moderate hypertonic stress in the present context (as a tonicity control for urea treatment) to activate Ras cannot exclude a modest role for Ras signaling, nor can it exclude Ras signaling at more pronounced degrees of osmotic stress or at later time points. Present data do, however, appear to eliminate an obligate role for Ras signaling in hypertonicity-inducible transcription through the ORE/TonE.

Ras dependence of Gadd153 expression has not previously been described. In the model of Ras- and ceramide-induced apoptosis in human leukemic cells, a Ras-dependent posttranslational modification (activation/phosphorylation) of Gadd153 was reported using a transfected dominant negative approach (3). Fan and Bertino (13) observed that overexpression of constitutively activated K-Ras may upregulate Gadd45, a related gene reported to be toxicity (but not urea) responsive (19).

The cell line(s) exhibiting inducible N17Ras overexpression identified through our selection process ap-
pears to be well suited to the present studies. Cell lines B7 (used for most studies) and B9 (used in corroboratory studies) exhibit a marked (≥10-fold; see below) increase in N17Ras expression after 24 h of dexamethasone induction. In all studies the potentially confounding effect of dexamethasone was eliminated through parallel treatment of control cells. Under no circumstances did dexamethasone treatment alone induce any detectable phenotypic changes with respect to the outcome measures examined in these studies (see below). The degree of basal N17Ras expression (the “leakiness” of the inducible expression system) could not be reliably estimated owing to the comigration of N17Ras and native Ras. (The precise degree of N17Ras overexpression could also not be reliably estimated for similar reasons.) Nonetheless, the uninduced N17Ras-transfected cells resembled native mIMCD3 cells with respect to all experimental outcome measures reported here (e.g., urea-inducible ERK activation, Egr-1 reporter gene expression, and Gadd153 expression). The solitary exception, unrelated to the urea response, was the greater response of the uninduced N17Ras transfectants to the heavy metal and oxidative stressor cadmium chloride (Fig. 8). Qualitatively, the N17Ras-B7 cell line proliferated somewhat more slowly than did control cells and exhibited greater sensitivity to thawing after cryopreservation (data not shown). With respect to functional data, inducible overexpression of N17Ras effectively inhibited known Ras-dependent events such as mitogen- and hypotonicity-associated ERK activation (Fig. 3). Therefore, it constituted a valid model in which to explore Ras-dependent aspects of the urea-inducible phenotype.

The timing of Ras-dependent signaling to ERK activation warrants comment. In contrast to the 15-min time point, Ras inhibition failed to block solute signaling to ERK at the 5-min time point. In aggregate, these data would suggest that the Ras-dependent effect on ERK activation peaks at 15 min whereas the Ras-independent effect peaks at 5 min. In general, ERK activation in response to potentially Ras-dependent mitogenic stimuli occurs quite early (e.g., 5 min). It is conceivable that in the present context an early (5-min) Ras-dependent signaling event leading to ERK activation is masked by a superimposed and maximal, yet transient, Ras-independent phenomenon, although data in support of this possibility are lacking.

The modest ability of inducible expression of N17Ras to blunt solute-inducible ERK activation, as assessed by anti-P-ERK immunoblotting (40–50% and only at 15 min; Fig. 4), contrasts with the ability of this stimulus to markedly inhibit urea-inducible Egr-1 reporter gene activity (Fig. 7). We previously hypothesized that the ability of urea to activate ERK accounted for urea-inducible Egr-1 transcription in its entirety, primarily on the basis of studies with the MAPK kinase (MEK) inhibitors PD-98059 and U-0126. These prior observations are not inconsistent with the present data. It is likely that at least two primary stimuli for ERK activation are operative in response to urea stress: one Ras dependent and (at least) one Ras independent. Both of these stimuli would appear to be MEK dependent on the basis of earlier data. Given the potential pleiotropic actions of urea, its ability to signal through multiple parallel pathways should not be surprising.

Interestingly, urea- and NaCl-inducible ERK activation at 15 min are approximately equivalently sensitive to Ras inhibition (Fig. 5). We previously showed that the mechanisms of ERK activation in response to NaCl (hypertonicity) and urea are distinct. The former is likely volume mediated, in contrast to that of urea, and the sensitivity (i.e., inhibition constant) of these phenomena with respect to MEK inhibitors differs by ≥1 log unit (35). Although it does not conflict with earlier data, the finding that these two solute stimuli should exhibit approximately equivalent sensitivity to Ras inhibition is nonetheless unexpected. The ability of Ras inhibition to partially block ERK activation in response to NaCl and the relative inability of NaCl to demonstrably activate Ras appear inconsistent. A modest effect of NaCl on Ras activation too subtle to achieve statistical significance, however, cannot be excluded (Fig. 1), nor can a modest effect of NaCl be excluded at a later time point.

Importantly, the effect of Ras inhibition on ERK activation may be more pronounced than indicated by Fig. 5. For rigorous analysis, statistical comparisons were only performed between different conditions in the same cell line (e.g., without and with dexamethasone in the N17Ras cells). Inspection of Fig. 4, however, and data from all similar experiments suggest that the ability of relatively low-dose urea and NaCl (200 mosmol/kgH₂O) treatment to induce ERK activation is blunted, even in the absence of dexamethasone induction. We speculate that this is a function of modest leakiness of the inducible expression system (5), in which a basal level of N17Ras expression is constitutively present. At higher osmolalities, in contrast, the responses of the transfected and untransfected cell lines were virtually identical. It could further be speculated that these data suggest a greater Ras dependence in the response to low-dose osmotic and urea stress; alternatively, the differential effect could be a manifestation of degree of activation alone, with the basal leakiness of N17Ras expression providing insufficient inhibition to blunt the signal generated in response to a robust osmotic stress.

Inducible expression of dominant negative-acting N17Ras failed to sensitize cells to the proapoptotic effect of elevated urea and NaCl concentrations. For these analyses, a higher concentration of urea (400 mM) than has been used in our signaling studies was required, because urea (200 mM) fails to exert a proapoptotic effect in this model (data not shown). Although associated with apoptotic induction in other models, in the mIMCD3 cell line, dexamethasone alone (in untransfected cells) failed to exert a proapoptotic effect (data not shown); therefore, it was unlikely to confound the present analysis. Previously, we suggested that a different receptor tyrosine kinase effector pathway, PI3K activation, may confer an element of resistance to osmotic and urea stress (38). Urea treatment and, to a


