Urea signaling to ERK phosphorylation in renal medullary cells requires extracellular calcium but not calcium entry

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Yang, Xiao-Yan, Hongyu Zhao, Zheng Zhang, Karin D. Rodland, Jean-Baptiste Roulet, and David M. Cohen. Urea signaling to ERK phosphorylation in renal medullary cells requires extracellular calcium but not calcium entry. Am J Physiol Renal Physiol 280: F162–F171, 2001.—The renal cell line mIMCD3 exhibits markedly upregulated phosphorylation of the extracellular signal-regulated kinase (ERK) 1 and 2 in response to urea treatment (200 mM for 5 min). Previous data have suggested the involvement of a classical protein kinase C (cPKC)-dependent pathway in downstream events related to urea signaling. We now show that urea-inducible ERK activation requires extracellular calcium; unexpectedly, it occurs independently of activation of cPKC isoforms. Pharmacological inhibitors of known intracellular calcium release pathways and extracellular calcium entry pathways fail to inhibit ERK activation by urea. Fura 2 ratiometry was used to assess the effect of urea treatment on intracellular calcium mobilization. In single-cell analyses using subconfluent monolayers and in population-wide analyses using both confluent monolayers and cells in suspension, urea failed to increase intracellular calcium concentration. Taken together, these data indicate that urea-inducible ERK activation requires calcium action but not calcium entry. Although direct evidence is lacking, one possible explanation could include involvement of a calcium-dependent extracellular moiety of a cell surface-associated protein.

fura 2; inner medullary collecting duct; Madin-Darby canine kidney; protein kinase C; hypotonicity

METHODS

Cell culture, experimental manipulations, and materials. Cells (mIMCD3) were grown in DMEM-F-12 in monolayers (32), and on achieving confluence they were serum deprived for 24 h before experimental manipulation unless otherwise noted. Cells were treated with the gentle dropwise addition of concentrated stocks of urea or NaCl to achieve the desired final osmolarity. Downregulation of classic PKC (cPKC) isoforms was achieved through 6- or 18-h pretreatment with 100 nM 12-O-tetradecanoylphorbol 13-acetate (TPA). For depletion of intra- and extracellular calcium, monolayers were

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taken out of serum for 24 h and treated with 1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid-acetone monoethyl ester (BAPTA-AM; 75 μM for 30 min), after which medium was washed twice with Hanks’ balanced salt solution (HBSS; 130 mM NaCl, 5 mM KCl, 1.5 mM CaCl₂, 1 mM MgCl₂, 20 mM HEPES, pH 7.4, 10 mM glucose) or calcium-free HBSS (130 mM NaCl, 5 mM KCl, 1 mM MgCl₂, 20 mM HEPES, pH 7.4, 10 mM glucose, 10 mM EDTA) and cells were incubated in either HBSS or calcium-free HBSS for 30 min before treatment with urea (200 mM for 5 min) or TPA (100 nM for 5 min). Inhibitors and activators of calcium transport and action (obtained from Calbiochem unless otherwise indicated) were used as follows: KN-93, 20 μM for 30 min; A-23187, 10 μM for 5–30 min; thapsigargin, 10–100 nM times indicated duration; epidermal growth factor (EGF, Sigma), 100 nM times indicated duration; dantrolene, 100 μM for 30 min; xestospongin C, 10 μM for 30 min; U-73122, 5–20 μM for 30 min; verapamil 10–100 μM for 30 min; nimodipine, 10 μM for 30 min; Nicl₃ (Sigma), 1–3 μM for 30 min; CdCl₂ (Sigma), 1–3 mM for 30 min; carboxyamidotriazole (CAI), 10 μM for 30 min; N,N,N',N'-tetraakis(2-pyrindylmethyl)ethylenediamine (TPEN; Sigma), 2 μM for 30 min; caffeine, 10 mM for 5 min; calmidazolium, 20 μM for 30 min; gadolinium chloride (Sigma), 20 μM-1 mM for 2–60 min; BAY K 8644, 10–100 μM for 30 min; ATP 10 μM for indicated interval; and bradykinin 100 nM for indicated interval. For titration of extracellular calcium (see Fig. 11), BAPTA concentration (BAPTA) necessary to establish indicated free calcium concentration (1.44, 2.33, and 5.43 mM BAPTA for 50%, 100%, and 300% free calcium, respectively) was estimated using MaxChelator (3) shareware (www.stanford.edu/~cpatton/maxc.html). Immunoblot experiments were performed at least twice with equivalent results; calcium ratiometry was performed at least three times per condition on 3 separate days with equivalent results.

Because it was observed that 30 min of calcium depletion approached the threshold of cell tolerance in terms of adherence, an alternative strategy was employed to partially dissociate the effects of adherence and calcium depletion. Cells were grown on collagen-treated dishes (BioCoat; Falcon/Becton-Dickinson 354400), which facilitate integrin-mediated cell binding to the epithelial cell surface. Confluent mIMCD3 or MDCK cells were grown to desired degree of confluence (70–100%) on 70–100% monolayers were pretreated with the cell membrane-permeant calcium chelator BAPTA-AM and placed in calcium-free medium. After a preequilibration period,

**Intracellular calcium measurement.** For population-wide analysis of cytosolic calcium mobilization, mIMCD3 and MDCK cells were grown to dense confluence on glass coverslips in DMEM-F-12 (Life Technologies). The cells were first rinsed twice with HBSS (in mM; 130 NaCl, 4.7 KCl, 1.18 MgSO₄ 15 HEPES, 1.25 CaCl₂, 5 glucose, pH 7.4). It was confirmed in independent experiments that this buffer was permissive for urea-inducible ERK activation for at least 6 h (data not shown). Cells were then loaded with fura 2-AM (2 μM) for 30 min at 37°C, followed by a 45-min washout in fura 2-free HBSS (37°C). Fluorescent emission was monitored at 510 nm with alternate excitation at 340 nm (F340) and 380 nm (F380) using a Hitachi F2000 spectrofluorometer (Hitachi Instruments, Naperville, IL); ratio (R) of F340/F380 is depicted in the figures. Alternatively, intracellular calcium concentration measurements were conducted on cell suspensions obtained by trypsinization of confluent cell monolayers. The suspended cells were loaded with fura 2 as previously described, washed twice with fura 2-free HBSS, and assayed for intracellular calcium concentration in a cuvette under constant, gentle agitation (2-ml final volume). Calibration of the fura 2 signal was performed using 20 μM of a 4:1 (vol/vol) mixture of Tris (pH 8.3, 1 M)-digitonin (2 g/l) and 20 μl EGTA (1 M) for Rₘₐₓ and Rₘᵢₐₙ, respectively, and a fura 2-calcium dissociation constant of 224 (7, 35).

For single-cell assessment of intracellular calcium release, a modification of the above protocol was used. mIMCD3 cells were grown to desired degree of confluence (~70–100%) on disposable glass-bottom 35-mm dishes (MatTek) and placed in serum-free medium for 24 h before fura 2-AM loading. Cells were loaded with fura 2-AM for 30 min and then placed in fura 2-AM-free calcium-containing HBSS for 20 min to permit ester cleavage. Cells were treated with 1 volume of 400 mM urea or with positive control (e.g., EGF 100 nM), and intracellular calcium was determined as previously described (33). Approximately 20 cells/field were individually and simultaneously analyzed per experiment.

**Calmodulin-dependent protein kinase II assay.** Calmodulin-dependent protein kinase II assay (CaM) kinase activity was measured in anti-CaM kinase II immunoprecipitates prepared from detergent lysates of monolayers. Briefly, cells were lysed in immunoprecipitation buffer (1% Triton X-100, 150 mM NaCl, 10 mM Tris, pH 7.4, 1 mM EDTA, 1 mM EGTA, 0.2 mM sodium orthovanadate, 0.2 mM phenylmethylsulfonyl fluoride, 0.5% NP-40) and immunoprecipitated with 2.5 μg of anti-CaM kinase II antibody (Transduction Laboratories) and protein A/G-bound agarose beads (Pharmacia) at 4°C for 1 h. Immunoprecipitates were washed three times with assay dilution buffer (20 mM MOPS, pH 7.2, 25 mM β-glycerol phosphate, 1 mM sodium orthovanadate, 1 mM dithiothreitol, 1 mM CaCl₂) and subjected to CaM kinase in vitro kinase assay in accordance with the manufacturer’s directions (Upstate Biotechnology, Lake Placid, NY, 17–135).

**RESULTS**

**Urea signaling to ERK activation is calcium dependent.** We sought to determine the role of extra- and intracellular calcium in the ability of urea to induce ERK phosphorylation in epithelial cells derived from the murine inner medulla. Confluent mIMCD3 cell monolayers were pretreated with the cell membrane-permeant calcium chelator BAPTA-AM and placed in calcium-free medium. After a preequilibration period,
cells were treated for 5 min with urea (200 mM) or the potent ERK activator TPA (100 nM). Calcium depletion did not affect the basal level of ERK phosphorylation or the level of ERK phosphorylation in response to the potent cPKC activator TPA (Fig. 1); however, in contrast, ERK phosphorylation in response to urea treatment was markedly inhibited by calcium depletion, suggesting the presence of a calcium-dependent step in urea signaling to this MAPK.

Urea-inducible ERK activation is not mediated by cPKC. Because urea-inducible ERK activation was calcium dependent, because urea treatment results in generation of IP_3 (11), and because PKC has been implicated broadly in downstream signaling events in response to urea (11), the role of classic PKC (classic calcium-dependent) isoforms in this phenomenon was investigated. Several complementary strategies were devised to establish or exclude a role for cPKC. In preliminary studies, the pharmacological PKC inhibitors staurosporine and calphostin C failed to suppress urea-inducible ERK phosphorylation (data not shown; concentrations and pretreatment intervals for these and other inhibitors are described in METHODS). Both of these inhibitors, however, induced ERK phosphorylation even in the absence of solute treatment so that no conclusion with respect to PKC dependence could be established. Protracted exposure to the PKC activator TPA was then used to downregulate PKC. In previous studies, 6 h of exposure to 100 nM TPA had proved sufficient for this purpose (11). Acute treatment with urea or TPA markedly increased ERK phosphorylation (Fig. 2). TPA pretreatment for 6 and for 18 h completely abolished the subsequent acute effect of TPA treatment but not that of urea treatment, implying the absence of dependence on cPKC (calcium-dependent) isoforms.

Because of the striking nature of this finding, in light of previous expectations with regard to urea signaling to ERK activation (11), translocation of calcium-dependent PKC isoforms was further examined as an additional correlate of PKC activation. After TPA or urea treatment, membrane and cytosolic fractions were prepared from mIMCD3 cells. Lysates were immunoblotted with antisera specifically recognizing the abundant calcium-dependent PKC isoform, PKC-α (anti-PKC-α), as well as an antibody broadly recognizing all major cPKC isoforms (monoclonal MC5). PKC-α is thus far the only cPKC identified in rodent inner medullary collecting duct (IMCD) (8). As anticipated, acute TPA treatment resulted in a decrease in PKC-α immunoreactivity in the cytosolic fraction and a corresponding increase in PKC-α immunoreactivity in the membrane fraction (Fig. 3). Similarly, TPA treatment resulted in a net decrease in cytosolic classical (MC5-immunoreactive) PKC isoform abundance and a corresponding net increase in the membrane fraction. This single band likely represented only PKC-α. In contrast, urea (200 mM) treatment failed to induce translocation of PKC-α or of any cPKC (MC5-immunoreactive) isoform. These data, including the lack of effect of PKC inhibitors, the lack of effect of PKC downregulation, and the absence of urea-inducible translocation of cPKC isoforms, strongly suggested that calcium-dependent PKC isoforms did not mediate the calcium-dependent effect of urea on ERK phosphorylation.

Urea-inducible ERK phosphorylation is not mediated by CaM kinase. A second calcium-dependent pathway to ERK activation is mediated via CaM kinase. Because CaM kinase activation has been shown to result in calcium-dependent ERK activation in at least two other contexts (12, 15), the role of this kinase in urea-inducible ERK phosphorylation was next explored. CaM kinase operates in a calmodulin-dependent fashion. The specific calmodulin inhibitor calmidazolium failed to block the effect of urea on ERK phosphorylation. In fact, the inhibitor activated ERK phosphorylation under both control and urea-treated conditions (data not shown). CaM kinases are effectively and specifically inhibited by the compound KN-93. This...
compound failed to inhibit urea-inducible ERK phosphorylation and influenced neither basal nor treated level of phosphorylation (data not shown). These data strongly suggested that urea-inducible ERK phosphorylation was not CaM kinase dependent. To corroborate these data, the effect of urea on CaM kinase activity was examined via in vitro (immune complex) kinase assay. Urea treatment failed to increase CAMKII activity (data not shown), whereas the effect of urea on the neural tissue-specific CAMKIV isoform was not examined in this renal epithelial cell line.

**Effect of depletion of intracellular calcium stores.** Although extracellular or intracellular calcium was essential for the ERK response to urea, as previously described, in some models calcium entry is dependent on intracellular calcium release. In addition, urea signaling results in activation of PLC-γ and generation of IP₃ (11). Therefore, it remained possible that the initiating event was calcium mobilization from an intracellular IP₃-sensitive calcium pool. Thapsigargin inhibits the calcium ATPase (SERCA) in the endoplasmic reticulum and thereby dissipates sequestered intracellular calcium stores. mIMCD₃ cells were pretreated with this compound for 30 min to deplete intracellular calcium stores before exposure to urea treatment. Thapsigargin pretreatment failed to block the ability of urea to increase ERK phosphorylation (Fig. 4A). The rapidity with which thapsigargin depletes intracellular calcium stores is model dependent and is a function of the rate of diffusion of calcium down its concentration gradient throughout the cytosol. To confirm that thapsigargin was effective and to eliminate the possibility that diffusion was too slow to result in depletion, an additional series of experiments was performed. Cells were briefly treated with thapsigargin (5 min); the ability of this short treatment to induce ERK phosphorylation was consistent with acute calcium release (Fig. 4B). EGF served as positive control for agonist-inducible ERK activation. EGF-inducible and thapsigargin-inducible calcium ERK phosphorylation were approximately equivalent. Even EGF treatment (to acutely empty calcium stores) in the presence of thapsigargin (to inhibit calcium reuptake) failed to abrogate subsequent urea-inducible ERK phosphorylation. It was therefore concluded that thapsigargin-sensitive intracellular calcium stores were not integral to the urea response leading to ERK activation. To further exclude this possibility, pharmacological inhibitors of calcium release pathways were examined. The inhibitor of IP₃-mediated calcium release, xestospongin C (16), failed to abrogate urea-inducible ERK phosphorylation. Similarly, the phospholipase inhibitor (and consequent inhibitor of IP₃ generation), U-73122 (4), also exerted no effect. Dantrolene and ruthenium red, inhibitors of the ryanodine-sensitive intracellular calcium pool, also failed to influence ERK phosphorylation in response to urea (data not shown). Several of these inhibitors may also act on other pathways involved in calcium metabolism (38); such considerations are only crucial when inhibition is observed and are irrelevant here because none of these inhibitors influenced urea signaling to ERK activation.

**Role of extracellular calcium.** To confirm that extracellular calcium was required for urea-inducible ERK activation, the effect of calcium depletion in the absence of intracellular calcium chelation was examined. Cells were placed in calcium-free medium and treated with urea or the calcium ionophore A-23187. Depletion of extracellular calcium alone modestly decreased the basal level of ERK phosphorylation and markedly abrogated the ability of both urea and the calcium ionophore A-23187 to induce ERK phosphorylation (Fig. 5A). Depletion of extracellular calcium also blocked the
ability of hypotonicity and the calcium ionophore ionomycin to induce ERK phosphorylation, but failed to inhibit the ability of EGF to activate ERK (Fig. 5B). These data are consistent with a requirement for extracellular calcium in the setting of ionophore and urea treatment and intracellular calcium in response to EGF. This distinction is significant because others have implicated activation of the EGF receptor as a key mediator in osmotic signaling in nonrenal cells (34). To further discriminate urea signaling from EGF signaling, additional studies were performed. Pretreatment with EGF downregulated EGF-inducible ERK activation without affecting urea-inducible ERK activation (Fig. 6A). In addition, pretreatment of cells with the tyrophostin inhibitor of EGF receptor signaling AG-1478 markedly attenuated EGF-inducible ERK activation but had no effect on urea-inducible ERK activation (Fig. 6B).

Extracellular calcium may enter via 1) voltage-gated calcium channels (including L-, T-, N-, P-, Q-, and R-types), 2) ionotropic glutamate receptor channels (including the N-methyl-D-aspartate (NMDA), kainate, and DL-amino-3-hydroxy-5-methylisoxazole-4-propronic acid (AMPA) receptors); 3) stretch-activated cation channels (including selective and nonselective types); and 4) so-called store operated calcium channels (also known as calcium release-activated channels), which respond to depletion of intracellular calcium. In addition, calcium may enter via the calcium exchanger, although it generally serves to export calcium against its concentration gradient. Of these pathways, only L-type calcium channels, stretch-activated calcium channels, store-operated calcium channels, and the calcium exchanger have been observed in renal epithelium. L-type calcium channels were implicated in calcium influx in other renal models of cell volume regulation [e.g., in response to cell swelling (23, 28) and hyperglycemia (13, 37)]. Verapamil, an inhibitor of L-type calcium channels, failed to significantly influence urea-inducible ERK phosphorylation (Fig. 7A). Other calcium channel blockers, including nifedipine and nimodipine, similarly exerted no effect (data not shown). BAY K 8644, an agonist of L-type calcium channels, only modestly activated ERK in the present model and only at very high concentration (100 μM, Fig. 7B). With respect to other calcium entry pathways, nickel, a blocker of T-type calcium channels, as well as CAI, an inhibitor of store-operated calcium channels, exerted no significant effect (data not shown). The inhibitor of stretch-activated calcium channels, gadolinium, failed to inhibit urea-inducible ERK activation, even at concentrations in the millimolar range (data not shown). Gadolinium or lanthanum also has been used nonspecifically to inhibit diverse calcium entry pathways; despite the conspicuous dependence of urea signaling on extracellular calcium, neither metal in concentrations to 10 mM affected the urea response (data not shown). Because of our inability to establish the route of calcium entry in response to urea, despite the conspicuous requirement for this ion, the kinetics of this potentially novel phenomenon were examined in greater detail. Interestingly, the ability of calcium depletion to abrogate urea signaling was a function of the calcium-free pretreatment interval (Fig. 8). Specifically, 5 min of calcium depletion modestly inhibited urea signaling, whereas 15 min produced a more profound inhibition. Essentially complete inhibition of urea signaling required 30 min of calcium depletion. In similar fashion, the ability of calcium repletion to restore urea responsiveness after extracellular calcium depletion was investigated. Interestingly, and consistent with data acquired through calcium depletion, calcium repletion also exhibited a time-dependent effect. Specifically, 10 min of calcium repletion exerted no effect, whereas at 1 and 4 h of repletion there was restoration of the ability of urea to activate ERK (Fig. 8).
9). Based on these data, it was initially surmised that calcium depletion might be disrupting cell adhesion to substratum and that calcium repletion might gradually permit re-establishment of normal architecture and hence calcium responsiveness. If this hypothesis were correct, then nonadherent mIMCD3 cells should fail to exhibit urea-inducible ERK activation. Treatment of cells with urea in the nonadherent (suspension) but calcium-replete state, however, did not appreciably influence the ability of urea to activate ERK (Fig. 10A). Therefore, the ability of calcium depletion to inhibit urea signaling was likely not a consequence of disrupted cell matrix adherence. To further explore this possibility, mIMCD3 cells were grown on a collagen substrate permitting greater (integrin-dependent) adherence in the absence of extracellular calcium. Cells grown under these conditions were substantially more tolerant of absent extracellular calcium; cells remained largely adherent beyond 6 h of calcium depletion. Even under these conditions, however, urea signaling to ERK activation was dramatically decreased at 0.5, 2, and 6 h of calcium depletion (Fig. 10B). Similar findings were observed with the renal epithelial MDCK cell line, which also exhibits urea-inducible ERK activation and was used for single-cell calcium analysis (see below).

The extracellular calcium concentration permissive for urea signaling to ERK was explored in greater detail. Specific reduction of extracellular calcium concentration with BAPTA indicated that 300 nM calcium was permissive for urea signaling (Fig. 11), whereas neither 30 nor 100 nM calcium was sufficient. In addition, to control for potential chelation of a noncalcium cation, pretreatment with the noncalcium-binding metal chelator TPEN was used. In the presence of 1 mM extracellular calcium, TPEN failed to abrogate urea signaling to ERK activation, underscoring the dependence of this phenomenon on calcium per se and not on a noncalcium (e.g., heavy metal) contaminant of the medium.

**Effect of urea on intracellular calcium.** These data strongly implicated calcium signaling in the mIMCD3 cell response to urea; however, inhibitor studies failed to identify a potentially responsible extracellular entry or intracellular release mechanism. Fura 2-based fluorescence ratiometric imaging was performed to definitively establish whether urea treatment resulted in intracellular calcium mobilization. Subconfluent monolayers of mIMCD3 cells were initially chosen for study because they are best suited to single-cell analysis. In this model, in the presence of extracellular calcium, EGF induced a marked increment in intracellular calcium (Fig. 12). Urea treatment, in marked contrast, failed to increase intracellular calcium. In some experiments (e.g., Fig. 12A), urea treatment actually suppressed F340/F380, an effect potentially attributable to volume-dependent quenching of the fura 2. Next, to complement single-cell analyses, studies aimed at detecting population-wide changes in intracellular calcium were performed. In the first model, mIMCD3 cells were grown to dense confluence on glass coverslips before loading with fura 2-AM. Under these conditions (and in the presence of extracellular calcium), whereas the positive controls ATP (14) and bradykinin rapidly mobilized intracellular calcium, urea failed to do so (Fig. 13, top). Because urea-inducible ERK activation also has been demonstrated in MDCK cells (42), which have been widely used for the study of agonist-inducible intracellular calcium release, studies were performed in parallel with this cell line. Again, the positive controls bradykinin (30) and ATP (29) induced a marked increase in intracellular calcium release,
Calcium may influence cell signaling via one of three general mechanisms: 1) intracellular release of stored calcium, 2) entry of extracellular calcium, and 3) extracellular calcium effect without calcium entry or release. These will be addressed in turn.

Calcium release. Based on prior data it was initially hypothesized that intracellular calcium release would mediate the effect of urea on ERK activation. Multiple calcium-dependent pathways of ERK activation have been described; the best characterized proceed through members of the Ras family of small G proteins (Ras and Rap) and include the following: 1) calcium-dependent activation of PYK2, leading to activation of SOS, a Ras guanine nucleotide exchange factor (GEF); 2) calcium- and diacylglycerol-dependent activation of the Ras-directed GEF, RasGRP; 3) calcium-calmodulin-dependent activation of the Ras-directed GEF, RasGRF; 4) CaM kinase-mediated inhibition of the Ras-directed GTPase-activating protein (GAP), SynGAP, leading to Ras activation; and 5) calcium-dependent activation of adenylate cyclase leading to cAMP-mediated activation of the Rap-directed GEF, cAMP-GEF (reviewed in Ref. 19). The first two mechanisms are operative in response to activation of receptor tyrosine kinases, events with which elements of urea signaling have been compared (11). It was anticipated that calcium-dependent urea signaling would operate through the first pathway because urea-inducible PYK2 (43) and Ras (40) activation have been shown previously in this model. Alternatively, because PKC has been implicated in urea signaling and because urea results in activation of PLC-γ and release of IP₃ (11), the second possibility also was considered. However, consistent with the inability of a dominant negative-acting Ras mutant to quantitatively inhibit urea-inducible ERK activation (40), calcium-dependent Ras activation was not responsible for urea signaling to ERK activation.

With respect to the role of PKC in urea signaling, further comment is warranted. The cPKC-independence of urea-inducible ERK activation (in contrast to urea-inducible immediate-early gene transcription (11]) was unexpected but convincingly demonstrated by complementary techniques. PKC activation in response to urea stress was suggested previously by the following observations: 1) inhibitors of PKC action blocked the ability of urea to activate transcription of the Egr-1 gene; 2) downregulation of PKC with chronic exposure to TPA blocked the ability of urea to activate Egr-1 transcription; and 3) urea treatment results in activation of PLC-γ and liberation of IP₃, hallmarks of production of the PKC activator diacylglycerol (11). The present data, which examine an experimental endpoint more proximal in the urea signaling cascade, do not support the involvement of cPKC in the early cell response to urea. Urea-inducible ERK activation was observed in mIMCD3 cells in suspension that were subjected to 5 or 15 min of urea treatment (200 mM for 5 min) in mIMCD3 and Madin-Darby canine kidney (MDCK) cell monolayers maintained on collagen-coated substrate to maximize adherence. For uniformity, a single empty lane between what are now lanes 2 and 3 of the MDCK blot has been digitally removed without influencing relative signal intensity among the remaining lanes.

Whereas urea failed to produce an effect (Fig. 13, bottom). In the second model of population-wide analysis of intracellular calcium release or entry, trypsinized cells in suspension were examined for a urea-inducible increment in intracellular calcium signaling in the presence of extracellular calcium. Again, whereas the positive controls bradykinin and ATP promptly increased intracellular calcium in both mIMCD3 and MDCK cell lines, urea failed to do so (data not shown). Data obtained from these three separate models strongly suggest that intracellular calcium mobilization, either from an extracellular source or intracellular stores, is not an element of early urea signaling and is not responsible for the calcium-dependence of urea-inducible ERK activation.

DISCUSSION

These data indicate that although the ability of urea to activate ERK in epithelial cells derived from the kidney medulla is dependent on extracellular calcium, neither calcium entry nor intracellular release of stored calcium is required for this effect. These findings raise several questions with respect to prior observations in this and related models and with respect to the mechanism of calcium action in urea signaling. Calcium may influence cell signaling via one of three general mechanisms: 1) intracellular release of stored calcium, 2) entry of extracellular calcium, and 3) extracellular calcium effect without calcium entry or release. These will be addressed in turn.

Fig. 10. Anchorage independence of urea-inducible ERK activation. A: degree of ERK phosphorylation evident in lysates prepared from mIMCD3 cells in suspension that were subjected to 5 or 15 min of sham treatment or urea (200 mM). B: effect of protracted calcium depletion (0–6 h) on ERK phosphorylation in response to urea treatment (200 mM for 5 min) in mIMCD3 and Madin-Darby canine kidney (MDCK) cell monolayers maintained on collagen-coated substrate to maximize adherence. For uniformity, a single empty lane between what are now lanes 2 and 3 of the MDCK blot has been digitally removed without influencing relative signal intensity among the remaining lanes.

Fig. 11. Extracellular calcium concentration permissive for urea signaling to ERK phosphorylation. Anti-phospho-ERK immunoblot of lysates prepared from control-treated cells (C) or cells treated with 200 mM urea for 5 min (U) in standard medium (containing 1 mM free calcium) or medium supplemented with BAPTA to achieve the indicated final free calcium concentration. Cells were also pretreated with the noncalcium-binding metal chelator TPEN (2 μM for 30 min; lanes 3 and 4), to control for nonspecific (noncalcium-binding) effects of BAPTA treatment.
not acutely influenced by PKC downregulation and did not induce translocation of cPKC isoforms.

In contrast to urea treatment, the role of PKC in anisotonicity has been explored previously in several contexts. Some (22, 25, 26, 36, 39) but not all (2, 17, 20) inhibitor-based and PKC downregulation studies have implicated PKC activation in hypertonicity-inducible signaling events and acquisition of the hypertonically stressed phenotype. PKC activation also has been directly observed in response to hypertonicity in cell culture models (26, 36). With respect to hypertonicity-inducible ERK activation specifically in the renal epithelial cell model, a number of investigators have indirectly implicated cPKC activation (22, 25, 39). Paradoxically, Preston et al. (31) showed that pharmacological activation of PKC inhibits events essential for acquisition of osmotic tolerance. Recently, Zhuang et al. (45) implicated both novel PKC and cPKC isoforms in ERK activation in response to hypertonicity in the nonrenal 3T3 cell model through a combination of inhibitor studies and direct assessment of PKC activation. In terms of ERK activation, however, 3T3 cells appear to be unresponsive to urea treatment (42).

Calcium entry. An extracellular, not intracellular, reservoir of calcium was essential for urea-inducible ERK activation. Multiple calcium entry pathways leading to ERK activation have been described, including L-type (voltage-gated) calcium channels, the NMDA receptor, and the Na\(^+\)/Ca\(^{2+}\) exchanger. Based on models of hyperglycemia (13, 37) and chronic renal failure with attendant uremia (24), it was hypothesized that L-type calcium channels may be responsible for the calcium-dependence of urea signaling. Indirect evidence from both inhibitor and activator studies strongly suggested that this pathway was not required for urea signaling to ERK activation (Fig. 7). Furthermore, the inability of urea to increase intracellular calcium as measured via fura 2 ratiometry virtually excluded this and other possible mechanisms of calcium entry.

Direct effects of extracellular calcium. Signaling events influenced by extracellular calcium in the absence of calcium entry are less well established. The cell membrane-associated calcium sensor responds directly to changes in ambient calcium concentration (5). In contrast to the present model, however, an effector arm of this G protein-coupled receptor influences intracellular calcium concentration (6). Cells interact with matrix and substratum via cell adhesion molecules (CAMs) of the integrin and syndecan family, and with each other via CAMs of the cadherin, selectin, and Ig-CAM families. Interactions mediated via cadherins...
and selectins are calcium dependent; only the cadherins appear to be expressed to a significant degree on renal epithelium. Increasing evidence supports a role for CAM interactions in mediating or modulating intracellular signaling events in contrast to the purely structural roles inferred earlier. Specifically, changes in cell adhesion dramatically influences ERK signaling in some models, an effect likely mediated at the level of MEK or Raf (1). This could account for the inability of Ras inhibition (upstream of Raf) to substantially abrogate urea signaling to ERK in the present model (40). Importantly, extracellular calcium depletion did not result in a global block to agonist-inducible ERK activation, as the ability of the peptide mitogen EGF to activate ERK was actually enhanced after removal of extracellular calcium (Fig. 5). In related fashion, calcium withdrawal induces loss of cell-cell contact, presumably at the level of the tight junction (21, 27). A consequence of this loss of cell-cell adhesion is a partial loss of cell polarization with respect to apical and basolateral membrane domains (41). Whether renal epithelial cell urea responsiveness is dependent on either of these two phenomena—calcium impingement on a CAM extracellular domain or on maintenance of cell polarization remains speculative; however, both are intriguing possibilities.

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