Rottlerin inhibits tonicity-dependent expression and action of TonEBP in a PKCδ-independent fashion

HONGYU ZHAO, WEI TIAN, AND DAVID M. COHEN
Division of Nephrology and Department of Cell and Developmental Biology, Oregon Health and Science University and the Portland Veterans Affairs Medical Center, Portland, Oregon 97201

Received 25 September 2001; accepted in final form 1 November 2001

Zhao, Hongyu, Wei Tian, and David M. Cohen. Rottlerin inhibits tonicity-dependent expression and action of TonEBP in a PKCδ-independent fashion. Am J Physiol Renal Physiol 282: F710–F717, 2002. First published November 13, 2001; 10.1152/ajprenal.00303.2001.—Novel protein kinase C (PKC) isoforms PKCδ and PKCe have recently been implicated in signaling by hypertonic stress. We investigated the role of the putative PKCδ inhibitor rottlerin on tonicity-dependent gene regulation. In the renal medullary mIMCD3 cell line, rottlerin blocked tonicity-dependent transcription of a tonicity enhancer (TonE)-driven luciferase reporter gene, as well as tonicity-dependent transcription of the physiological tonicity effector gene aldose reductase, but not urea-dependent transcription. Consistent with these data, rottlerin inhibited tonicity-dependent expression of TonE binding protein (TonEBP) expression at the mRNA and protein levels. Another inhibitor of both novel and conventional PKC isoforms, GF-109203X, suppressed TonEBP-dependent transcription but failed to influence tonicity-inducible TonEBP expression. Global PKC downregulation with protracted phorbol ester treatment, however, failed to influence tonicity-dependent signaling, arguing against a PKCδ-dependent mechanism of rottlerin action in this model. In addition, hypertonic stress failed to induce phosphorylation of PKCδ. Furthermore, in a PC-12 cell model with a comparable degree of tonicity-dependent transcription, constitutive overexpression of dominant negative-acting PKCδ or PKCe effectively decreased tonicity signaling to extracellular signal-regulated kinase activation, as expected, but failed to influence TonE-dependent transcription. TonE-dependent transcription, however, remained sensitive in this PC-12 cell model. In the aggregate, these data indicate that rottlerin dramatically inhibits tonicность-dependent TonEBP expression and TonE-dependent transcription but, despite its reputed mode of action, does so through a PKCδ-independent pathway.

hypertonicity; inner medullary collecting duct; renal; kidney; signal transduction

THE 11 PROTEIN KINASE C (PKC) ISOFORMS INCLUDE CONVENTIONAL (α, β1, β2, γ), NOVEL (δ, ε, η, and θ), AND ATYPICAL (ζ and λ) ISOFORMS (27). THROUGH A COMBINATION OF PROMINENTLY INHIBITOR-BASED AND PKC ДOWNSREGULATION STUDIES, SEVERAL GROUPS HAVE BROADLY IMPLICATED PKC ACTIVATION IN HYPERTONIC STRESS SIGNALING AND IN THE ACQUISITION OF THE HYPERTONICALLY STRESSED PHENOTYPE (16, 20, 21, 33, 36); OTHER GROUPS, HOWEVER, HAVE FAILED TO OBSERVE PKC DEPENDENCE OR ACTIVATION IN THIS SETTING (2, 9, 10). IN A RECENT AND THOROUGH SERIES OF EXPERIMENTS, ZHUANG ET AL. (45) DESCRIBED A ROLE FOR THE NOVEL PKC ISOFORMS PKCδ AND PKCe IN OSMOTIC ACTIVATION OF THE EXTRACELLULAR SIGNAL-REGULATED KINASE (ERK), MITOGEN-ACTIVATED PROTEIN KINASE (MAPK) (45). IN THEIR FIBROBLASTIC 3T3 CELL MODEL, HYPERTONIC STRESS INDUCED MEMBRANE TRANSLLOCATION OF PKCδ AND PKCe, AS WELL AS THE CONVENTIONAL PKCє ISOFORM.

In addition to activating the ERKs, however, hypertonic stress activates the p38 and c-jun N-terminal-directed kinase (JNK) families of MAPKs through activation of their respective upstream activators (reviewed in Ref. 18). The role of these events in the downstream activation (41) of the tonicity enhancer binding protein (TonEBP), the ubiquitous tonicity-responsive transcription factor of higher eukaryotes (26), and in TonEBP-dependent transcription (19, 32) remains incompletely understood. TonEBP was extensively and elegantly characterized in the present physiological context by Kwon et al. (reviewed in Ref 13) and cloned independently as TonEBP (26), nuclear factor of activated T cells-5 (23), and osmotic response element binding protein (17). In response to hypertonic stress, TonEBP upregulation occurs at the mRNA (40) and protein levels (26), as well as at the level of subcellular localization (41). Signaling events influencing expression and translocation of TonEBP are receiving increasing attention. Signaling through the p38 MAPK (32) and proteasomal function (41) have been implicated in tonicity-dependent transcription or tonicity-dependent TonEBP expression; however, urea, the renal medullary solute, blocks tonicity-dependent expression of TonEBP independently of these pathways (38). Downstream effectors of TonEBP in the renal epithelium, physiologically stressed in vivo by dramatic fluctuations in ambient tonicity, include the sorbitol-synthesizing enzyme aldose reductase and transporters of osmotically active solutes (reviewed in Ref 3).

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

http://www.ajprenal.org

Address for reprint requests and other correspondence: D. M. Cohen, Mailcode PP262, Oregon Health Sciences Univ., 3314 S.W. US Veterans Hospital Rd., Portland, OR 97201 (E-mail: cohend@ohsu.edu).
We investigated the role of novel PKCδ and PKCe isoforms in tonicity-dependent gene regulation and observed that, whereas the purportedly PKCδ-specific inhibitor rottlerin (12) potently inhibited the tonicity-dependent increase in TonEBP expression at the mRNA and protein levels and inhibited TonEBP-dependent transcription of both a TonE-driven luciferase reporter gene and the physiological TonEBP effector aldose reductase, it did so in a PKCδ-independent fashion.

METHODS

General methods. Cell culture and solute treatment were performed as previously described (30, 37). The following inhibitors and stimuli (purchased from Calbiochem unless otherwise indicated) were used: 200 mM urea (Sigma); 100 mM NaCl (200 mosmol/kg H2O; Sigma); 100 mM epidermal growth factor; 100 mM 12-O-tetradecanoylphorbol 13-acetate (TPA; Sigma); 10–50 μM rottlerin; 30–300 mM Go-6983, 10 μM GF-109203X, 1 μM Go-6976, and 50 μM SB-203580. Cells receiving pretreatment with an inhibitor or solute (i.e., urea) remained exposed to the pretreatment compound for the duration of the experiment until determination of the experimental end point. Depicted data represent means ± SE unless indicated; statistical significance is assigned to P < 0.05 (Excel; Microsoft). PC-12 cell lines expressing dominant negative-acting PKC isoforms were kindly provided by Drs. Thomas McMahon and Robert O. Messing and were previously described (15). Cell lines were as follows: vector controls, DND-21; and DN-δ, originally designated DND-21; and DN-ε, originally designated DNE-1.

TonEBP and aldose reductase RNase protection assay. Total cellular RNA was prepared using the Trizol reagent (Life Technologies) in accordance with the manufacturer's directions. Murine TonEBP and aldose reductase partial cDNAs were previously described (38). Plasmids were linearized with XhoI, gel-purified (QIAEX II kit; Qiagen), reconstituted in diethylpyrocarbonate-treated H2O, and used for biotinylated antisense riboprobe preparation with biotin RNA labeling mix and T7 polymerase (Boehringer Mannheim). Hybridization of probe (800 pg) with total RNA (15 μg) was performed at 45°C (RPA II kit; Ambion); subsequent digestion with RNase A and T1 was performed in solution at 37°C. Detection was achieved with the BrightStar BioDetect nonisotopic detection kit (Ambion).

Reporter gene assay. The plasmid BGT-2X-Luc, based on the TonE element (25), has been previously described (37). Cells were transfected with the plasmid of interest (as well as a lacZ expression plasmid under the control of the cytomegavirus long terminal repeat) via electroporation; luciferase and β-galactosidase activities were quantitated at 6 h of treatment as previously described (6) except that, for the latter, a Luminescent β-Galactosidase Detection Kit II (Clontech) was used in accordance with the manufacturer's directions.

Immunoblotting. Immunoblotting, using commercially available anti-phospho-ERK and anti-phospho-p38 (Cell Signaling Technologies) was performed as previously described (42). Anti-phospho-(Thr505) PKCδ was obtained from Cell Signaling Technologies and used in accordance with the manufacturer's directions. Rabbit polyclonal anti-TonEBP (anti-N-terminal NFAT5) was kindly provided by Drs. Cristina Lopez-Rodriguez and Anjana Rao (23) and used at 1:600 dilution in 0.075% Tween 20. For TonEBP immunoblotting, a combined mechanical and detergent-based lysis system (7) was employed to ensure nuclear disruption.

RT-PCR. RT-PCR was performed essentially as previously described, using primers selected to be specific for PKCδ, PKCe, PKCζ, and PKCa isoforms and the PKC-related kinase-1 (PRK1). Primers were chosen from variable regions that lacked significant homology with other known PKC isoforms. For each isoform depicted (see Fig. 9), a product of only the anticipated size was generated. Optimization for each isoform was performed by varying buffer composition and pH over known increments for a total of 12 individual reactions/sample (OptiPrime; Stratagene) in accordance with the manufacturer's directions. Primer pairs for PKC isoforms (Integrated DNA Technologies, Coralville, IA) were as follows (all are listed 5’ to 3’): PKCδ, TGC TAT GGG-3’ and TCA CTT TGT AGG CCC CAG CAG CAC ACC AAG GGT GTC GTC TTC; PKCe, TGG CCT GCA CC-3’ and TCA CTT TGT AGG CCC CAG CAG CAC ACC AAG GGT GTC GTC TTC; PKCζ, TGG CCT GCA CC-3’ and TCA CTT TGT AGG CCC CAG CAG CAC ACC AAG GGT GTC GTC TTC; PKCa, TGG CCT GCA CC-3’ and TCA CTT TGT AGG CCC CAG CAG CAC ACC AAG GGT GTC GTC TTC; PKCε, TGG CCT GCA CC-3’ and TCA CTT TGT AGG CCC CAG CAG CAC ACC AAG GGT GTC GTC TTC.

RESULTS

Because Zhuang et al. (45) observed dependence of elements of tonicity-mediated signaling on PKCδ in murine fibroblasts, we examined whether other aspects of tonicity signaling also require this pathway. In the best-studied model of tonicity-dependent gene regulation, transcription proceeds through upregulated interaction of TonEBP with its cognate DNA consensus element within the 5’-flanking region of a tonicity-responsive gene (reviewed in Ref. 13). mIMCD3 cells were transiently transfected with BGT-2X-Luc (44), an expression plasmid harboring a luciferase reporter gene under the control of two tandem repeats of the BGT TonE enhancer element (35) (Fig. 1A). As anticipated and consistent with the data of others, hypertonic stress increased TonE-dependent transcription by approximately eightfold; this effect was markedly inhibited by pretreatment of cells with the PKCδ-specific inhibitor rottlerin (12) and the nPKC and conventional (c)PKC inhibitor GF-109203X (Fig. 1B). Cytomegalovirus long terminal repeat-driven β-galactosidase reporter gene activity, used for normalization, was inhibited by <20% in the presence of either NaCl or rottlerin. We were unable to assess the effect of these inhibitors on basal and tonicity-dependent transcription from an enhancerless thymidine kinase promoter because this construct exhibited no detectable activity in the present model. Therefore, the effect of these inhibitors on urea-inducible transcription of the immediate-early gene Egr-1 (Fig. 1C) was examined in parallel. Consistent with earlier observations (6), urea increased Egr-1 reporter gene activity by approximately sixfold; however, neither GF-109203 nor rottlerin significantly inhibited this effect (Fig. 1D), underscoring the specificity of the effect on tonicity-dependent signaling. Next, similar studies were performed in the more physiological context of tonicity-dependent (and TonEBP-
dependent) transcription of the physiological osmotic stress effector aldose reductase (Fig. 2). Hypertonic stress (100 mM NaCl/H2O 6 h) markedly increased aldose reductase expression at the mRNA level, and this effect was substantially inhibited by pretreatment with rottlerin. GF-109203X failed to block the effect of hypertonicity, as did the specific inhibitor of cPKC isoforms Go-6976. These data implicated an inhibitory effect of rottlerin on TonEBP action, and this pathway was examined further.

The effect of rottlerin on tonicity-dependent TonEBP expression at the mRNA level was examined via an RNase protection assay, as previously described (38). Consistent with our earlier data and those of others, hypertonic stress increased TonEBP mRNA expression severalfold; this effect was almost completely blocked by rottlerin (Fig. 3A). GF-109203X, however, appeared to be ineffective in this assay. The effect of these nPKC inhibitors on TonEBP expression at the protein level was explored in parallel. As anticipated and again consistent with the data of others (26, 40), hypertonic stress modestly increased TonEBP protein abundance in whole cell lysates (Fig. 3B). Again, strikingly similar to the mRNA expression data, rottlerin markedly inhibited basal and tonicity-inducible TonEBP expression, whereas GF-109203X failed to do so.

PKCβ and PKCε have been implicated in signaling to ERK activation by hypertonicity (45). Because the putative PKCβ inhibitor rottlerin blocked NaCl signaling to TonE-dependent transcription, the effect of another maneuver designed to abrogate PKCβ was examined. Phorbol ester activates all classic and nPKC isoforms, but not the atypical PKCζ and PKCλ isoforms (28). Protracted treatment with TPA has been used to down-regulate all of the TPA-responsive PKC isoforms; this treatment effectively downregulated tonicity-dependent signaling to ERK activation in 3T3 cells (45). In the

Fig. 1. Rottlerin inhibits tonicity-dependent transcription in a tonicity enhancer (TonE)-dependent reporter gene. mIMCD3 cells were transiently transfected with BGT-2X-Luc (A) or Egr-1-Luc derived from the proximal 1.2 kb of the murine Egr-1 5'-flanking sequence (6, 39; C) and then pretreated with the indicated inhibitor for 60 min before exposure to hypertonic stress (100 mM NaCl × 6 h; B) or urea stress (200 mM × 6 h; D). Fold-induction by solute, relative to control treatment for each pretreatment pair, is indicated at the base of each bar. Gal, β-galactosidase; TK, thymidine kinase promoter; AP-1, activator protein-1; SREs, serum response elements; Emin, Egr-1 minimal promotor. †P < 0.05 relative to vehicle pretreatment.
mIMCD3 cell model, hypertonic NaCl markedly increased ERK phosphorylation, as previously shown by us and others (2, 43), but protracted pretreatment with TPA failed to inhibit this effect (Fig. 4A). In similar fashion, the effect of PKC downregulation on tonicity-dependent transcription was examined. It was anticipated that this rottlerin-sensitive process should be sensitive to PKC downregulation. Unexpectedly, yet consistent with the lack of effect of PKC downregulation on tonicity-dependent ERK activation, prolonged TPA treatment failed to significantly influence TonE-mediated transcription (Fig. 4B). As a positive control to confirm PKC downregulation (5), the effect of urea on transcription of the Egr-1 gene was substantially suppressed by this maneuver (Fig. 4B).

Activation of the p38 MAPK has been implicated in TonEBP-dependent transcription, and a role for PKCδ in tonicity-inducible ERK activation has been suggested by data obtained in nonrenal cells (45). For these reasons, the effect of rottlerin pretreatment on tonicity-dependent ERK and p38 activation/phosphorylation was examined in mIMCD3 cells. Rottlerin exhibited a negligible effect on ERK and p38 activation under basal conditions (Fig. 5, A and B). ERK activation by hypertonicity was in large part sensitive to rottlerin, whereas ERK activation by the phorbol ester TPA and by the peptide mitogen epidermal growth factor was not (Fig. 5A). MAPK p38 was substantially activated only by hypertonic stress; TPA and epidermal growth factor produced no consistently demonstrable effect (Fig. 5B). Rottlerin pretreatment only very modestly inhibited p38 activation in response to hypertonic stress. It was concluded in preliminary fashion that rottlerin was not inhibiting TonEBP signaling via an effect on p38. To corroborate these data, the effect of rottlerin was compared with that of the p38 inhibitor SB-203580 with respect to inhibition of tonicity-dependent TonEBP-mediated transcription. In mIMCD3 cells transiently transfected with BGT-2X-Luc, the effect of SB-203580 was very modest whereas the effect of rottlerin was again substantial (Fig. 5C). In the aggregate, these data indicated that rottlerin was not operating through inhibition of a p38-dependent pathway in the present context.

Data from Zhuang et al. (45) support a role for PKCδ in tonicity signaling. Our own data suggested that PKCδ...
might be involved, as it is a rottlerin-sensitive process, whereas other data showed that toxicity signaling was insensitive to PKC downregulation. Additional studies were undertaken to determine the role of PKCδ activation in the toxicity-inducible transcription. A series of PC-12 cell lines were used that constitutively overexpress dominant negative-acting truncation mutants of nPKCδ and nPKCe isoforms or empty vector alone. These cell lines exhibited dramatically downregulated PKCδ and PKCe activity in other experimental contexts (8, 14, 15, 34). Initially, a degree of hypertonic stress was established at which toxicity-dependent transcription could be demonstrated in this PC-12 model. Hypertonic stress (800 mosmol/kgH2O NaCl) increased ERK dual phosphorylation at 15 min of treatment in untransfected PC-12 cells (Fig. 6A), consistent with earlier observations (24). A comparable degree of induction was observed in vector-transfected PC-12 cells; however, in the PC-12 cell lines harboring dominant negative PKCδ and PKCe, there was a marked diminution in the effect of hypertonicity in the absence of an effect on basal levels of ERK activation (Fig. 6A). There was no effect on p38 activation in response to hypertonicity. It was concluded that the dominant negative PKC isoforms were effectively inhibiting nPKC-dependent signaling in this model, consistent with the findings of Zhuang et al. (45) in the 3T3 cell line.

Next, the role of nPKC in toxicity-dependent transcription, in contrast to ERK activation, was examined in this validated PC-12 cell model. Cells were transfected with a luciferase reporter gene, under the control of tandem copies of the TonE enhancer element cloned upstream of a thymidine kinase promoter, and then exposed to hypertonic NaCl for 6 h. In both wild-type PC-12 cells and in PC-12 cells stably transfected with vector alone, toxicity-dependent transcription was increased approximately six- to sevenfold relative to control (Fig. 6B), consistent with observations with this construct in other cell lines (38). Interestingly, in the cell lines expressing dominant negative PKCδ and PKCe, there was no inhibitory effect on TonE-mediated transcription (expressed as fold-induction), arguing against a role for nPKC in this process. (Of note, there was substantial variation in the basal level of TonE-dependent transcription between cell lines whereas the fold induction remained unchanged.) To corroborate that rottlerin was effective in this model as well, both wild-type and dominant negative PKCδ-expressing PC-12 cell lines were exposed to hypertonicity, in the presence or absence of rottlerin pretreatment. Again, as in the mIMCD3 cell model, rottlerin effectively abrogated toxicity-dependent transcription in both wild-type and dominant negative nPKCe-expressing PC-12 cell lines (Fig. 6C).

Phosphorylation accompanies activation of PKC isoforms; the effect of hypertonic stress on phosphorylation of PKCδ (Thr505) was examined in the mIMCD3 Fig. 6. Effect of rottlerin on toxicity-dependent transcription is independent of novel isoforms PKCδ and PKCe. A: the PC-12 cell model was used to establish the contribution of novel PKC isoforms to toxicity-dependent signaling. The ability of hypertonic stress to activate ERK phosphorylation was examined in control PC-12 cells and in cell lines overexpressing dominant-negative (DN) PKCδ, DN-PKCe, or empty vector alone. The effect of control treatment (C) or hypertonic stress (H) on ERK phosphorylation (filled arrowheads) in each of these cell lines is depicted in the top panel; the effect on p38 phosphorylation via anti-P-p38 immunoblotting is depicted in the bottom panel. B: effect of hypertonic stress on TonE-dependent transcription of a luciferase reporter gene in PC-12 cells and in PC-12-based stably transfected cell lines. Despite a substantial decrement in basal (Control) TonEBP-dependent transcription between cell lines whereas the fold induction remained unchanged.) To corroborate that rottlerin was effective in this model as well, both wild-type and dominant negative PKCδ-expressing PC-12 cell lines were exposed to hypertonicity, in the presence or absence of rottlerin pretreatment. Again, as in the mIMCD3 cell model, rottlerin effectively abrogated toxicity-dependent transcription in both wild-type and dominant negative nPKCe-expressing PC-12 cell lines (Fig. 6C).

Phosphorylation accompanies activation of PKC isoforms; the effect of hypertonic stress on phosphorylation of PKCδ (Thr505) was examined in the mIMCD3
subjected to immunoblotting with anti-P-(Thr505) PKC indicated concentration of NaCl or with TPA (100 nM) and then or from cells treated for the indicated interval (in min) with the
Whole cell detergent lysates were prepared from control-treated cells
RNA prepared from mIMCD3 cells to determine which
cell model as an additional correlate of PKC
Fig. 7. Hypertonic stress fails to activate PKC in mIMCD3 cells. Whole cell detergent lysates were prepared from control-treated cells or from cells treated for the indicated interval (in min) with the indicated concentration of NaCl or with TPA (100 nM) and then subjected to immunoblotting with anti-P-(Thr505) PKCδ.

In the aggregate, these data strongly supported the absence of a role for PKCδ in tonicity signaling to ERK activation and TonE-dependent transcription in renal medullary cells. Atypical isoforms PKCζ and PKCλ (PKCζ is the murine homolog of PKCδ) are insensitive to downregulation with protracted TPA treatment; it was therefore hypothesized that one of these isoforms may be involved in rottlerin-sensitive tonicity signaling. RT-PCR analysis was performed with poly-A+ RNA prepared from mIMCD3 cells to determine which isoforms were expressed in these cells. PKCδ, PKCε, PKCζ, and PKCλ, as well as the related kinase PRK1, were all expressed in renal medullary cells (Fig. 8). Of the atypical isoforms, only PKCζ has been widely studied. In addition, only PKCα, PKCδ, and PKCζ appear to be widely expressed. Furthermore, because PKCζ is not downregulated by protracted TPA treatment and does not necessarily undergo translocation in the setting of activation, it was a reasonable candidate for mediating the effect of hypertonicity on TonE-dependent transcription. The inhibitor Go-6983 is reported to inhibit PKCζ (11); however, this compound failed to abrogate the effect of hypertonic NaCl on tonicity-dependent transcription by reporter gene assay (data not shown). The only PKC isoform unaffected by Go-6983 is PKCθ, more commonly known as protein kinase D (PKD) (31). The cPKC-directed inhibitor Go-6976 is also a potent PKCθ inhibitor (11), yet it failed to influence TonE-dependent transcription (Fig. 2 and data not shown).

**DISCUSSION**

Recent evidence supports a role for PKCδ in hypertonic stress signaling. Zhuang et al. (45) described the sensitivity of tonicity-inducible ERK activation to both the putative PKCδ-specific inhibitor rottlerin and to PKC downregulation (45). In addition, membrane translocation of the PKCδ isoform was detected. Although no other data have implicated nPKC isoforms per se, others have described an inhibitory effect of PKC downregulation (affecting both novel and conventional isoforms) on hypertonic signaling to ERK activation (16, 20, 21, 33, 36). We attempted to extend this observation to transcriptional events engendered by hypertonic stress and uncovered a discrepancy between rottlerin sensitivity and PKCδ dependence. Rottlerin dramatically suppressed TonEBP-dependent transcription, apparently by inhibiting the tonicity-dependent increment in TonEBP expression at the mRNA and protein level. Interestingly, another PKCδ inhibitor, GF-109203, also suppressed tonicity-mediated transcription but failed to abrogate TonEBP expression. Most importantly, through a combination of biochemical and dominant-negative approaches, hypertonicity-inducible TonEBP-mediated transcription was found to be independent of PKCδ or PKCe activation.

It is puzzling that two inhibitors with purportedly similar mechanisms of action should operate at distinct loci in the same signaling pathway. It is possible that the effect of rottlerin and GF-109203X overlap in terms of TonEBP-dependent transcription but that rottlerin exhibits an additional and independent effect on TonEBP expression. GF-109203X reportedly exhibits greater potency with respect to conventional PKC isoforms than novel ones; nonetheless, inhibitors of classic PKC (e.g., Go-6976 and PKC downregulation with protracted TPA treatment) failed to influence TonEBP-dependent signaling. It is also possible that regulation of the TonE-dependent reporter might differ in some respects from that of the native aldose reductase gene.

Beyond PKCδ, present data also refute a role for any known PKC isoforms in mediating tonicity-de-

![Fig. 8. Renal medullary cells express other atypical and related PKC isoforms. Poly-A+ RNA from mIMCD3 was subjected to RT-PCR using primers specific for PKCδ, PKCe, PKCζ, and PKCλ, and the PKC-related kinase PRK1 (see METHODS); resultant products were resolved, stained with ethidium bromide, and visualized by fluorescence. PCR products of the expected size were detected for each of the 5 kinases. The order of the gel lanes has been digitally modified, and several intervening lanes have been deleted; however, all lanes are derived from the same gel and are depicted at the same scale and exposure. L, ladder.](image-url)
pendent transcription in renal medullary cells. PKC downregulation with prolonged phorbol ester treatment failed to influence TonEBP-dependent signaling; hence, the cPKC (α, β1, βII, and γ) and nPKC (δ, ε, η, ζ) isoforms are unlikely to be involved. The atypical isoforms PKCζ and PKCλ may be TPA insensitive, and both are expressed in mIMCD3 cells (data herein and Ref. 4); the former isoform, unlike TonEBP-dependent transcription, is sensitive to Go-6983, whereas few data are available regarding PKCλ translocation or inhibitor sensitivity. PKCμ (PKD) should be sensitive to Go-6976, whereas TonEBP-dependent transcription is not. PRK1, the related kinase, is expressed in mIMCD3 cells; however, as for PKCλ, limited data are available with respect to pharmacological inhibition. In the aggregate, these data argue against a role for any PKC isoform in this process in renal medullary cells, although involvement of PKCλ or a PKC-related kinase cannot be fully excluded on the basis of available data and reagents. This is in marked contrast to the observations of Zhuang et al. (45), who noted, in the fibroblastic 3T3 cell model, that both rottlerin and protracted phorbol ester treatment abrogated tonicity-dependent signaling to ERK activation, findings consistent with bona fide involvement of PKC.

These observations with rottlerin extend the small list of pharmacological inhibitors known to influence TonEBP-dependent signal transduction. Activation of the p38 MAPK has been variably implicated in TonEBP expression or function (19, 32) as has proteasomal processing (41); inhibition of these pathways abrogates TonEBP-dependent signaling. We have shown that pretreatment with the membrane-permeant renal medullary solute urea (200 mM), but not the permeable solute glycerol, blocks hypertonic stress-inducible TonEBP mRNA expression and TonEBP-dependent transcription (38). The mechanism through which rottlerin is acting in the present context, independently of PKCζ, is unclear. Rottlerin blocks TonEBP mRNA expression at an early time point and, because the tonicity-dependent increase in TonEBP mRNA is not a consequence of altered mRNA stability (40), rottlerin would appear to block TonEBP transcription. No data are available with regard to signaling events related to TonEBP transcription per se.

Although rottlerin does not appear to mediate its effect on tonicity-dependent signaling through PKC, we demonstrated expression of PKCδ, PKCe, PKCζ, and PKCλ, as well as the related PRK1 kinase, in the mIMCD3 murine inner medullary cell line. These data are consistent with and extend the observations of others. Ostlund et al. (29) detected renal expression of PKCa, PKCδ, PKCe, and PKCζ via a combination of approaches. Aristimuno and Good (1) noted immunodetectable PKCe, PKCβ1, PKCδ, PKCe, and PKCζ in the inner stripe of the outer medulla and medullary thick ascending limb, as well as agonist-inducible membrane translocation of PKCe. Chou et al. (4), in contrast, examining inner medullary collecting duct suspensions, noted agonist-inducible translocation of only the PKCζ isoform. In this latter model, which likely bears the closest relation to the mIMCD3 cell culture system, expression of PKCa, PKCe, PKCe, PKCζ, and PKCλ was demonstrated via immunoblotting (4).

In summary, the putative PKCζ-specific inhibitor, rottlerin, blocks tonicity-dependent transcription and tonicity-dependent expression of the TonEBP transcription factor, but does so in a PKCζ-independent fashion. These data establish a potentially useful pharmacological inhibitor of TonEBP expression and TonEBP-dependent signaling and call into question the presumed specificity of rottlerin action.

The authors thank Thomas McMahon and Robert O. Messing for the PC-12 stable transfecants and Cristina Lopez-Rodriguez and Anjana Rao for the anti-NFAT5/TonEBP antisum.

This work was supported by National Institute of Diabetes and Digestive and Kidney Diseases Grant DK-52494 and by the Department of Veterans Affairs.

REFERENCES


14. Hundle B, McMahon T, Dadgar J, Chen CH, Mochly-Rosen D, and Messing RO. An inhibitory fragment derived from protein kinase C epsilon prevents enhancement of nerve growth