Hyperosmolality induces activation of cPKC and nPKC, a requirement for ERK1/2 activation in NIH/3T3 cells

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Zhuang, Shougang, Syu-Ichi Hirai, and Shigeo Ohno. Hyperosmolality induces activation of cPKC and nPKC, a requirement for ERK1/2 activation in NIH/3T3 cells. Am. J. Physiol. Cell Physiol. 278: C102–C109, 2000.—Protein kinase C (PKC) has been reported to be associated with the activation of extracellular signal-regulated kinase (ERK) by hyperosmolality. However, it is unclear whether hyperosmolality induces PKC activation and which PKC isoforms are involved in ERK activation. In this study, we demonstrate that NaCl increases total PKC activity and induces PKCα, PKCβi, and PKCδ translocation from the cytosol to the membrane in NIH/3T3 cells, suggesting that hyperosmotic stress activates conventional PKC (cPKC) and novel PKC (nPKC). Further studies show that NaCl-inducible ERK1 and ERK2 (ERK1/2) activation is a consequence of cPKC and nPKC activation, because either downregulation with 12-O-tetradecanoylphorbol 13-ace-tate or selective inhibition of cPKC and nPKC by GF-109203X and rottlerin largely inhibited the stimulation of ERK1/2 phosphorylation by NaCl. In addition, we show that NaCl increases diacylglycerol (DAG) levels and that a phospholipase C (PLC) inhibitor, U-73122, inhibits NaCl-induced ERK1/2 phosphorylation. These results, together, suggest that a hyperosmotic NaCl-induced signaling pathway that leads to activation of ERK1/2 may sequentially involve PLC activation, DAG release, and cPKC and nPKC activation.

sodium chloride; phospholipase C; diacylglycerol; phosphorylation; novel and conventional protein kinase C; extracellular signal-regulated kinase-1 and -2

CELLS IN THE RENAL MEDULLA are normally exposed to an extremely hyperosmotic milieu caused by accumulation of high levels of NaCl and urea during the process of urinary concentration (4). A change in osmolality due to cellular hydration under the influence of hormones, nutrients, and oxidative stress has been observed in all other cell types studied so far (17). The cells react to increased osmolality with activation of ion transport in the plasma membrane, alterations in metabolic processes, and induction of gene transcription and protein phosphorylation (17). Over the past two decades, the regulation of these events in response to osmotic changes had become much more thoroughly understood. However, it is largely unknown which intracellular signals activate or control these osmolality-regulatory responses and, especially, regulate gene expression. It has been proposed that transcription factors might be involved in a coordinated program of gene expression governing adaptation to hyperosmotic stress (9). Recently, Cohen and Gullans (9) demonstrated that high concentrations of urea and NaCl can increase expression of two immediate-early gene transcription factors, c-Fos and Egr-1, in tissues of renal cells [Madin-Darby canine kidney (MDCK) and LLC-PK1]. Expression of c-Fos was also detectable in nasal gland tissue from ducklings and neuronal tissue from rats in response to hyperosmotic stimulation (13, 18, 32).

Because the activation of transcription factors is usually regulated by distinct signaling pathways, some recent studies have focused on the identification of signaling molecules involved in the osmotic regulation of transcription factor expression. In renal inner medullary collecting duct cells (mMCD3), it has been shown that hyperosmotic urea-induced transcription of Egr-1 is mediated by extracellular signal-regulated kinase (ERK) (8). Hypotonicity-mediated transcriptional regulation of this gene is also partially involved in ERK activation in this cell type (39). The pathway leading to activation of ERK can be triggered by a variety of stimuli, including osmotic stress, and has been well elucidated in growth factor-stimulated cells: ERK is directly activated by mitogen-activated protein kinase kinase (MAPK kinase or MEK), and MEK is activated by Raf-1 kinase; Raf-1 is recruited to the membrane, where it is activated by Ras (25). Activation of ERK by hyperosmotic stress is reported to be mediated by Raf/MEK (33). However, it appears that Ras does not function as an activator of Raf in this case, because blocking Ras activation with its negative mutant, Ras-Asn17, did not affect ERK activation in response to hyperosmotic stress (7).

In addition to Ras, another recognized upstream activator of Raf is protein kinase C (PKC) (5, 6, 21). PKC is a serine/threonine kinase, and PKC isoforms have been divided into three categories on the basis of their structure and biochemical properties: conventional PKC (cPKC), including PKCα, PKCβi, PKCβII, and PKCγ; novel PKC (nPKC), including PKCδ, PKCe, PKCθ, and PKCλ; and atypical PKC (apPKC), including PKCζ and PKCβi/δ. cPKC and nPKC are modulated by phorbol ester and are also activated by diacylglycerol (DAG), whereas apPKC is activated by phosphatidylinositol 3,4,5-trisphosphate [PtdIns(3,4,5)P3]. Endogenous DAG is derived from phosphatidylinositol 4,5-bisphos-
phosphate following hydrolyzation by phospholipase C (PLC). This process is accompanied by production of d-myoinositol 1,4,5-trisphosphate ([Ins(1,4,5)]<sub>29</sub>), whereas PtdIns(3,4,5)<sub>3</sub>, PKCa) are expressed in this cell line was used because both cPKC and nPKC by hyperosmotic NaCl in NIH/3T3 mouse fibroblasts. It has been shown that PKC plays a central role in the activation of ERK by a wide array of stimuli (1, 16, 41). Involvement of PKC in transducing hyperosmotic signals leading to activation of the ERK pathway was also demonstrated by use of PKC inhibitors in MDCK cells (33). However, it is unclear whether hyperosmotic stress activates PKC and which PKC isoforms are involved in the ERK activation in response to this stress.

In this study, we investigated the effect of hypertonicity on PKC activation and assessed the roles of cPKC and nPKC in activation of ERK1 and ERK2 (ERK1/2) by hyperosmotic NaCl in NIH/3T3 mouse fibroblasts. This cell line was used because both cPKC and nPKC isoforms (PKCa, PKCb, and PKCc) are expressed in this cell type (26) and because these three members have been shown to activate a Raf-1/ERK pathway in fibroblasts (5, 6, 21). Our results show that hyperosmotic NaCl increases total PKC activity and induces association of PKCa, PKCb, and PKCc with cell membranes. Furthermore, we demonstrate that ERK1/2 activation by NaCl is dependent on PLC and cPKC/nPKC-mediated signaling.

**MATERIALS AND METHODS**

Materials. 12-O-Tetradecanoylphorbol 13-acetate (TPA) and platelet-derived growth factor (PDGF) were from R&D Systems. Ly-294002 and U-73122 were purchased from Biomol Research Lab. GF-109203X and rottlerin were purchased from Alexis Biochemicals. A PKC assay kit was purchased from Gibco, and the DAG assay kit was from Amersham. Rabbit polyclonal anti-MAPK R2 (CT) and sheep polyclonal anti-rabbit PKCα (3147SA), and PKCβ and PKCε were purchased from New England Biolabs. Anti-rabbit PKCα (3195SA), PKCb (3147SA), and PKCc (13198-0150) antibodies were obtained from Life Technologies. NaCl was from Fisher Scientific.

Cell culture. NIH/3T3 cells and NIH/3T3 cells overexpressing PKCa, PKCb, and PKCc were maintained in DMEM supplemented with 7% calf serum. Cells at 70–80% confluence were grown arrested by incubation in 0.5% calf serum DMEM for 24 h before use. For hypertonic treatment, NaCl stock solution was added to the medium at the appropriate concentration.

Transient transfection and immunofluorescence microscopy. Transient transfection and immunofluorescence were carried out as described previously (40). Briefly, NIH/3T3 cells were seeded on plates with coverslips and transiently transfected by the calcium phosphate precipitation method with 6 µg of expression vector for full-length mouse PKCa, PKCb, or PKCc. After starvation, cells were stimulated with 0.6 M NaCl for 5 min and then fixed with 3% (wt/vol) paraformaldehyde in PBS and permeabilized with 0.1% Triton X-100. The coverslips with cells were incubated with respective anti-PKC rabbit monoclonal antibodies, followed by visualization with anti-rabbit IgG FITC-labeled antibodies. Mounted cells were observed under a fluorescence microscope (Nikon, Optiphot photo 2 EF D2). In our previous report, we showed that the disappearance of dark nuclei is a diagnostic parameter indicating PKC translocation from the cytoplasm to the membrane (27).

Establishment of cell lines overexpressing PKC isoforms. NIH/3T3 cells seeded on 10-cm dishes (2 × 10<sup>5</sup> cells) were transfected with 1 µg of pSv2Neo and 10 µg of expression vector for PKCa, PKCb, or PKCc by the calcium phosphate coprecipitation method (39). After 2 days of culture in 7% calf serum DMEM, cells were selected with G418 (Genetec; Gibco) at a concentration of 300 µg/ml. After 7 days of culture in the selection medium, colony-forming cells were picked up and subcultured. The expression level of PKC isozymes for each subculture was tested by Western blotting, and cell lines overexpressing PKCa, PKCb, or PKCc were used for further experiments.

In vitro kinase assay. The total PKC activity was determined by a PKC assay system following procedures provided by Gibco BRL. Briefly, treated or nontreated cells on 10-cm dishes were washed with ice-cold PBS and harvested in extraction buffer (20 mM Tris, pH 7.5, 0.5 mM EDTA, 0.5 mM EGTA, 0.5% Triton X-100, and 25 µg/ml each aprotinin and leupeptin). After homogenization, the homogenate was incubated on ice for 30 min and centrifuged for 5 min at 6000 g. The supernatant was used for detection of PKC activity with a PKC-specific substrate, the synthetic amino-terminal acetylated peptide corresponding to amino acids 4–14 of myelin basic protein (Ac-MBP(4–14)) (38). The PKC-specific activity (pmol/min) per assay tube was obtained after subtraction of nonspecific activity (pmol/min) per assay tube with PKC inhibitor peptide and normalized to total protein.

Western blot analysis. Lysates were prepared and subjected to electrophoresis in 10% SDS-polyacrylamide gel. After transfer of protein to a polyacrylifene membrane, the membranes were blocked with 5% (wt/vol) nonfat dry milk in 1× PBS overnight at 4°C. PKCa, PKCb, and PKCc were detected with their respective antibodies. Total ERK1/2 was detected by a rabbit anti-rat ERK antibody (anti-MAPK R2), and phosphorylated ERK1/2 was detected by phosphospecific ERK1/2 antibodies. The antibody-antigen complexes were detected using horseradish peroxidase-conjugated anti-rabbit or anti-mouse IgG and visualized by a standard chemiluminescence method performed according to the manufacturer’s instructions.

Cell fractionation. Cell fractionation was performed as described previously (40). Briefly, cells were washed with ice-cold PBS and harvested in lysis buffer. The lysate was sonicated for 20 s and then centrifuged at 1,300 g for 10 min. Supernatant was collected and centrifuged at 100,000 g for 40 min. The cytosol fraction was harvested, and the pellet was solubilized in cold lysis buffer containing 1% Nonidet P-40 and then centrifuged at 10,000 g for 10 min. This supernatant was used as the membrane fraction. Equal amounts of protein (20 µg/lane) were resolved by SDS-PAGE for subsequent immunoblot analysis.

DAG assay. sn-1,2-DAG was measured with a DAG assay reagent system following the instructions provided by Amersham. Briefly, treated cells (1 × 10<sup>6</sup>) were washed once with ice-cold PBS without Ca<sup>2+</sup> and Mg<sup>2+</sup> and harvested in 1 ml of PBS. After centrifugation for 5 min at 1,500 rpm and 4°C, the cell pellet was resuspended in 200 ml of cold PBS. Lipids were extracted, and sn-1,2-DAG was radiolabeled using DAG kinase and [γ-<sup>32</sup>P]ATP. The labeled sn-1,2-DAG was separated by TLC and counted by scintillation counter (Beckman, LS 3801) for 4 min. The amount of sn-1,2-DAG was calculated.
from the mean counts per minute for each of the triplicate tubes.

Statistical analysis. Data were analyzed by the paired two-tailed Student’s t-test.

RESULTS

Comparison of ERK1/2 activation by hyperosmotic stress and TPA in NIH/3T3 Cells. It has been reported that hyperosmotic stress induces ERK activation in several cell lines, including MDCK, 3Y1, and PC-12 cells (20, 33). TPA is a strong activator of cPKCs and nPKCs and also induces ERK1/2 activation in a number of cell systems. To understand the role of PKC in mediating ERK1/2 activation by hyperosmotic stress in NIH/3T3 cells, we compared the activation of ERK1/2 by hyperosmotic NaCl and TPA. ERK1/2 activation was measured by Western blotting with a phosphospecific ERK1/2 antibody. When cells were incubated with 0.6 M NaCl, ERK1/2 phosphorylation was detectable at 5 min and maximal at 25 min (Fig. 1A). However, TPA (100 ng/ml) stimulated a very rapid increase in ERK1/2 phosphorylation that reached a maximum level within 5 min (Fig. 1A). Notably, the total cellular ERK1/2 level remained constant through all of these time courses, as shown by Western blotting with an antibody for ERK1/2 that detects both the phosphorylated and unphosphorylated forms of the kinases. Densitometric analysis revealed that the extent of ERK1/2 phosphorylation 25 min after NaCl incubation was similar to that in TPA-treated cells at this time point (~7-fold relative to control; Fig. 1B). The ERK activity induced by either NaCl or TPA was sustained for at least 60 min with only a slight decrease (data not shown). These results indicate that both hyperosmotic stress and TPA strongly activate ERK1/2 in NIH/3T3 cells but that the kinetics of ERK1/2 activation by hyperosmotic stress are slower than those of activation by TPA.

ERK1/2 activation by hyperosmotic stress depends on cPKC and nPKC. cPKC and nPKC, but not aPKC, are sensitive to phorbol ester. Chronic treatment of cells with TPA can downregulate or deplete cPKC and nPKC isoforms. In NIH/3T3 cells, PKCα is abundantly expressed, and PKCβ, PKCe, and PKCd are also detectable (26, 40). Previously, we showed that incubation of cells with TPA (200 ng/ml) for 24 h could deplete all of PKCβ, and most of PKCα and PKCe without affecting PKCd (40). To investigate whether cPKC and nPKC isoforms are required for ERK1/2 activation by hyperosmotic stress, we preincubated cells with TPA for 24 h and then exposed them to hyperosmotic NaCl. As shown in Fig. 2, the prolonged TPA treatment completely abolished the activation of ERK1/2 by readdition of TPA, verifying that downregulation of PKC is effective. Under this condition, the phosphorylation of ERK1/2 by 0.6 or 1.0 M NaCl was also largely inhibited, suggesting that certain isoforms of the cPKC and nPKC groups do mediate activation of ERK1/2 induced by hyperosmotic stress.

To further evaluate the role of cPKCs and nPKCs in activation of ERK1/2 by hyperosmotic stress, we examined effects of two PKC inhibitors on hyperosmotic NaCl-induced ERK1/2 phosphorylation. GF-109203X selectively inhibits cPKC isoforms, with similar po-
tency on PKCα, PKCβI, PKCβII, and PKCy subtypes (34), whereas rottlerin is a more selective inhibitor for nPKCs, including PKCδ and PKCe isoforms (14). As shown in Fig. 3, A and C, treatment of cells with rottlerin caused a dose-dependent inhibition of NaCl-stimulated ERK1/2 phosphorylation, with maximal effect at 25 µM. Accordingly, 10 µM rottlerin also gave a large inhibition of the phosphorylation of ERK1/2 by TPA (Fig. 3, B and D). Although lower concentrations of GF-109203X (1–5 µM) had essentially no effect on NaCl stimulation of ERK1/2 phosphorylation, at 10 µM this agent significantly inhibited the phosphorylation of ERK1/2 by NaCl, which corresponds to its inhibition, to a lesser extent, of TPA-stimulated ERK1/2 phosphorylation (Fig. 3, A–D). More importantly, a combination of two inhibitors led to additional inhibition of stimulation of ERK1/2 by NaCl (Fig. 3, A and D). These data suggest that hyperosmotic stress-induced ERK1/2 activation is mediated by both groups of PKC isoforms, and nPKCs may contribute more than cPKCs in NIH/3T3 cells.
Induction of PKC activity by hyperosmotic stress. To test whether hyperosmotic stress activates PKCs, we first measured total PKC activity in vitro using Ac-MBP(4–14) as a substrate. As shown in Fig. 4, incubation of cells with 0.6 M NaCl caused a 2.6-fold increase in PKC activity compared with untreated cells at 10 min. This indicates that hyperosmotic stress can induce PKC activation.

We next examined which members of cPKC and nPKC groups are activated by hyperosmotic stress. To do this, we transiently overexpressed PKC\(\alpha\), PKC\(\delta\), and PKC\(\epsilon\) in NIH/3T3 cells and then checked the translocation of PKC\(\alpha\), PKC\(\delta\), and PKC\(\epsilon\) by immunofluorescence, since PKC activity is induced by phospholipid binding and translocation of PKC from the cytosol to the membrane following a certain stimulus has generally been considered to represent its activation (28). As shown in Fig. 5A, overexpressed PKC\(\alpha\), PKC\(\delta\), and PKC\(\epsilon\) were localized in the cytosol of untreated cells. After treatment with hyperosmotic NaCl, all three PKC isoforms quickly translocated to the membrane, resulting in the disappearance of dark nuclei. The percentage of cells with translocated PKCs was 52% in those overexpressing PKC\(\alpha\), 48% in those overexpressing PKC\(\delta\), and 36% in those overexpressing PKC\(\epsilon\). TPA-induced PKC translocation was more evident than NaCl-induced translocation and was seen in all cells overexpressing individual PKC isoforms. These results suggest that hyperosmotic stress can activate all the members of cPKC and nPKC groups present in NIH/3T3 cells.

The translocation of these PKC isoforms from the cytosol to the membrane was further confirmed by Western blotting using cell fractionation from NIH/3T3 cells stably overexpressing various PKC isoforms. As shown in Fig. 5B, before stimulation, PKC\(\alpha\) and PKC\(\epsilon\) were distributed in both cytosol and membrane fractions, whereas PKC\(\delta\) was found solely in the cytosol fraction. Exposure of cells to 0.6 M NaCl led to a rapid increase in the levels of various PKC isoforms in the membrane fraction, with PKC\(\delta\) and PKC\(\epsilon\) clearly seen as early as 2 min and PKC\(\delta\) detectable at 5 min. The abundance of individual isoforms in this fraction was further augmented with increasing incubation time after treatment, peaking at 25 min. Corresponding to the increase in the membrane fraction, the levels of various PKCs in the cytosol were decreased, indicating that these PKC isoforms translocated from the cytosol to the membrane following treatment.

Production of DAG following hyperosmotic stress. Because activation of cPKC and nPKC is a consequence of DAG production following PLC activation in intact cells, we assessed the ability of hyperosmotic NaCl to stimulate DAG production using TLC. Figure 6 shows that treatment of cells with hyperosmotic NaCl led to an increase in DAG production in a dose-dependent manner, with a 3.5-fold increase in DAG levels in the cells incubated with 1 M NaCl. Similar results were seen in sorbitol-treated cells (data not shown), suggesting that hyperosmotic stress induces PLC activation.
PLC inhibitor, but not PI-3 kinase inhibitor, blocks the activation of ERK1/2 by hyperosmotic stress. Because PKCa, PKCd, and PKCe are major intermediates in the ERK1/2 activation pathway and because hyperosmotic stress also stimulates an increase in DAG levels, we next examined whether the activation of PLC underlies the hyperosmolality-induced activation of ERK1/2. As shown in Fig. 7, A and B, when cells were treated with U-73122, a potent inhibitor of both PLC-γ and -b (31), ERK1/2 phosphorylation stimulated by hyperosmotic NaCl was severely inhibited, whereas only a small inhibitory effect was seen in cells treated with LY-294002, a specific inhibitor for PI-3 kinase. Similarly, U-73122 but not LY-294002 partially inhibited PDGF-stimulated ERK1/2 phosphorylation. In contrast, there was no inhibition of TPA-induced phosphorylation of ERK1/2 by either inhibitor. These results suggest that hyperosmotic stress-induced ERK1/2 phosphorylation is critically dependent on a PLC-initiating signal transduction pathway in NIH/3T3 cells.

**DISCUSSION**

In this report, we provide evidence that members of the cPKC and nPKC groups are responsible for a major portion of the hyperosmotic stress-stimulated signal transduction that leads to the activation of ERK1/2. We also demonstrate that hyperosmotic stress induces an increase in total PKC activity and translocation of PKCa, PKCd, and PKCe from the cytosol to the membrane after the stimulation of NIH/3T3 cells with NaCl.

The mechanism for PKC activation by growth factors is well established in mammalian cells, and our findings show that a partly common mechanism is also operating in hyperosmotic stress-induced PKC activation. Stimulation of tyrosine kinase receptors activates PLC and PI-3 kinase, leading to an increase in DAG and PtdIns(3,4,5)P3 levels, which in turn mediate activation of PKC (10, 29). Here we show that treatment of cells with hyperosmotic NaCl results in an increase in DAG levels (Fig. 6). Furthermore, it has been previously reported that hyperosmotic NaCl increases release of Ins(1,4,5)P3 and that urea induces PLC phosphorylation (30, 33). These results suggest that hyperosmotic stress triggers PLC activation, resulting in release of DAG and Ins(1,4,5)P3 and, as a consequence, PKC activation. It is likely that PLC and cPKC/nPKC lie along the same pathway, which mediates the activation of ERK1/2 by NaCl, because NaCl-induced ERK1/2 phosphorylation can be completely

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**Fig. 6.** Hyperosmotic NaCl increases diacylglycerol (DAG) level. Cells were left untreated or treated with NaCl for 10 min at various concentrations as indicated. sn-1,2-DAG levels were determined by DAG assay kit as described under MATERIALS AND METHODS (n = 3, means ± SE).

**Fig. 7.** Effect of U-73122 and LY-294002 on hyperosmotic NaCl-stimulated ERK1/2 phosphorylation. Cells were pretreated with vehicle (0.1% DMSO), 5 µM U-73122, or 10 µM LY-294002 for 20 min and then left untreated (C) or treated for 25 min with 0.6 M NaCl (N), 30 ng/ml PDGF (P), or 100 ng/ml TPA (T). A: phosphorylated ERK1/2 (p-ERK1, p-ERK2) and total ERK1/2 were analyzed by Western blot as described in Fig. 1. Blots are representative of 5 independent experiments. B: immunoblotted phosphorylated ERK1/2 levels were quantified by densitometry, and data were expressed as percentage of induction relative to phosphorylated ERK1/2 level stimulated by NaCl alone. Results are means ± SE of 5 independent experiments. *P < 0.05; **P < 0.01.
inhibited by either blocking PLC or TPA downregulation.

How PLC is activated by osmotic stress is currently unknown. Because the activation of PLC is a consequence of phosphorylation of tyrosine kinase receptors or activation of G protein-coupled receptors, one possibility is that osmotic stress induces the activation of these receptors, which then lead to activation of PLC. This notion is supported by two studies, one of which shows that hyperosmotic sorbitol stimulates the aggregation of epidermal growth factor receptors (30) and the other of which shows that urea-induced Egr-1 expression that is mediated by PLC/PKC can be inhibited by genistein, a potent tyrosine kinase inhibitor (10). However, Terada et al. (33) showed that ERK activation by NaCl is insensitive to genistein, which argues against tyrosine growth factor receptors being involved in this process. Another possibility is that a non-membrane receptor/sensor-mediated mechanism may be involved in the activation of PLC/PKC. Recently, it was reported that a decrease in cell volume induces tyrosine phosphorylation of several proteins (12) and that the activation of PKCα can be triggered by dehydration caused by hyperosmotic stress in an in vitro system (12). Thus it is possible that shrinkage of cells by extracellular hyperosmolality exerted by a nonpermeable solute such as NaCl may directly activate these signaling molecules. Further studies are needed to examine these two possibilities.

Among ten PKC isoforms reported, members of the cPKC and nPKC groups seem to play the most essential roles in the activation of ERK1/2 by hyperosmotic NaCl. This is suggested by several results obtained in this work, as follows. First, the downregulation of PKC by prolonged TPA treatment inhibits most of the phosphorylation of ERK1/2 by 0.6 or 1.0 M NaCl (Fig. 2). Second, highly selective cPKC and nPKC inhibitors abrogate ERK1/2 phosphorylation (Fig. 3). Third, treatment of cells with NaCl increases total PKC activity and induces association of PKCα, PKCδ, and PKCe with the membrane (Figs. 4 and 5). Using immunostaining analysis, we also observed αPKC (PKCα and PKCζ) translocation following NaCl treatment (data not shown). However, these isoforms may not play a major role in NaCl-stimulated ERK1/2 activation, because the activation of ERK1/2 was severely reduced when cPKC and nPKC were downregulated, leaving αPKCs intact (Fig. 2), whereas inhibition of PI-3 kinase, an upstream activator of αPKC, by LY-294002 had only a limited effect on NaCl-induced ERK1/2 phosphorylation (Fig. 7). Although our data suggest that members of cPKC and nPKC groups present in NIH/3T3 cells mediate NaCl-induced ERK1/2 activation, the contribution of individual PKC isoforms to activation of these kinases remains to be defined.

The mechanism of PKC in activating ERK is currently being studied. Increasing evidence indicates that PKC directly activates Raf-1 and that Raf-1 then activates ERK through MEK. Kolch (21) reported that PKCα phosphorylates Raf-1, both in vitro and in vivo. Cacace et al. (5) demonstrated that overexpressing PKCe in R6 rat fibroblasts resulted in a marked increase in Raf-1 and MEK activity, and Cai et al. (6) also showed that PKCe was able to directly activate Raf-1 in vitro. Recently, we reported that another nPKC member, PKCδ, activates MEK in a Raf-dependent manner (35). These results, together with our finding that NaCl treatment increases total PKC activity and induces PKCα, PKCδ, and PKCe translocation, suggest that these PKC isoforms may activate ERK1/2 through a Raf/MEK pathway in cells exposed to hyperosmotic stress.

The physiological significance of the activation of the ERK pathway by osmotic stress in mammalian cells is still not clear. However, in yeast, evidence has been presented for a role of MAPK pathways in regulation of the transcriptional activation of the glycerol synthetic pathway in response to high-salt conditions (2). In MDCK cells, PKC-mediated ERK1/2 activation seems not to be required for transcriptional stimulation of two osmolyte transporter genes, myo-inositol and betaine, as osmolyte transporter mRNA accumulation is still stimulated by hypertonicity after PKC depletion (23). Nevertheless, the possibility cannot be ruled out that the PKC-ERK pathway may mediate the expression of other osmoregulatory genes by inducing transcription factors such as Egr-1 and c-Fos (8, 9, 13, 18, 32). On the other hand, it has been shown that Na+-myo-inositol cotransporter and Na+-K+-Cl cotransporter contain several PKC phosphorylation sites (24, 37) and that the activation of PKC results in an increase of their activities (11, 15). Activation of Na+-myo-inositol cotransporter is required for uptake of inositol, an organic solute that plays an important role in adaptive regulation of long-term hyperosmolality, whereas activation of Na+-K+-Cl cotransporter increases intracellular K+ and Na+, which initiate signals for the induction of genes responsible for organic osmolyte accumulation (3, 4). The functional roles of PKC isoforms in regulation of these cotransporters remains unexplored. The finding that PKCα, PKCδ, and PKCe are activated in response to hyperosmotic NaCl lays the groundwork for further study of PKC-mediated signaling events involving regulation of cotransport systems, as well as the hypertonic activation of genes.

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