Phospholipase C-γ1 is involved in signaling the activation by high NaCl of the osmoprotective transcription factor TonEBP/OREBP

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High NaCl elevates activity of the osmoprotective transcription factor TonEBP/OREBP by increasing its phosphorylation, transactivating activity, and localization to the nucleus. We investigated the possible role in this activation of phospholipase C-γ1 (PLC-γ1), which has a predicted binding site at TonEBP/OREBP-phospho-Y143. We find the following. (*i*) Activation of TonEBP/OREBP transcriptional activity by high NaCl is reduced in PLC-γ1 null cells and in HEK293 cells in which PLC-γ1 is knocked down by a specific siRNA. (*ii*) High NaCl increases phosphorylation of TonEBP/OREBP at Y143. (*iii*) Wild-type PLC-γ1 coimmunoprecipitates with wild-type TonEBP/OREBP but not TonEBP/OREBP-Y143A, and the coimmunoprecipitation is increased by high NaCl. (*iv*) PLC-γ1 is part of the protein complex that associates with TonEBP/OREBP at its DNA binding site. (*v*) Knockdown of PLC-γ1 or overexpression of a PLC-γ1-SH3 deletion mutant reduces high NaCl-dependent TonEBP/OREBP transactivating activity. (*vi*) Nuclear localization of PLC-γ1 is increased by high NaCl. (*vii*) High NaCl-induced nuclear localization of TonEBP/OREBP is reduced if cells lack PLC-γ1, if PLC-γ1 mutated in its SH2C domain is overexpressed, or if Y143 in TonEBP/OREBP is mutated to alanine. (*viii*) Expression of recombinant PLC-γ1 restores nuclear localization of wild-type TonEBP/OREBP in PLC-γ1 null cells but not of TonEBP/OREBP-Y143A. (*ix*) The PLC-γ1 phospholipase inhibitor U72133 inhibits nuclear localization of TonEBP/OREBP but not the increase of its transactivating activity. We conclude that, when NaCl is elevated, TonEBP/OREBP becomes phosphorylated at Y143, resulting in binding of PLC-γ1 to that site, which contributes to TonEBP/OREBP transcriptional activity, transactivating activity, and nuclear localization.

hypertonicity | phosphorylation | proteomics

High NaCl activates TonEBP/OREBP (also named NFAT5), a Rel-family transcription factor that is central to osmoprotection (1). The resultant increase in transcription of its target genes causes accumulation of osmoprotective organic osmolytes and heat shock proteins. Interstitial fluid in the renal medulla has high NaCl, which energizes urinary concentration. TonEBP/OREBP protects renal medullary cells from this high NaCl and also contributes to urinary concentration by transactivating genes that code for urea transporters and aquaporin 2.

High NaCl rapidly increases phosphorylation of TonEBP/OREBP (1). There are numerous possible phosphorylation sites in TonEBP/OREBP, including 216 serines, 15 tyrosines, and 111 threonines. Because phosphorylation generally contributes to activity of transcription factors, it seemed likely that high NaCl–induced phosphorylation of TonEBP/OREBP contributes to its increased activity. In support of this idea, numerous kinases have been shown to be involved in high NaCl–induced activation of TonEBP/OREBP, including protein kinase A, ataxia telangiectasia mutated (ATM), phosphatidyl 3-kinase class IA, Fyn, MEKK3, and p38 (1). Each is necessary for full activation of TonEBP/OREBP, but none alone is sufficient. Despite these findings, there is little information about which specific amino acids in TonEBP/OREBP are phosphorylated by high NaCl.

The present studies were initiated when we noted that phosphorylated tyrosine 143 (Y143) in TonEBP/OREBP is a predicted binding site for phospholipase C-γ1 (PLC-γ1) (Minimotif Miner; http://mnm.engr.uconn.edu/MNM/SMSSearchServlet). Sites in target proteins that are phosphorylated by tyrosine kinases can become binding sites for PLC-γ1 through its Src homology 2 domains (SH2-N and SH2-C). PLC-γ1 is known to signal heat stress (2) and oxidative stress (3) and can also signal hypertonic stress, for example regulating ERK1/2 (4) and glucose transport (5, 6). Although hypertonicity activates PLC-γ1 lipase activity, the lipase activity per se is not required for stimulation of glucose transport (5).

With this in mind we directly measured phosphorylation of TonEBP/OREBP at Y143 and binding of PLC-γ1 to that site. We used a transcriptional reporter assay to test the effect of knocking down PLC-γ1 on high NaCl–induced increase of TonEBP/OREBP transcriptional activity. Also, because high NaCl increases transcriptional activity of TonEBP/OREBP by increasing its transactivating activity and by localizing it to the nucleus (1), we looked for a possible effect of PLC-γ1 on both of those functions.

Results

PLC-γ1 Contributes to High NaCl–Induced Increase of the Transcriptional Activity of TonEBP/OREBP. Transcriptional activity of TonEBP/OREBP was measured using an ORE-X luciferase reporter in mouse embryonic fibroblasts (MEFs) deficient in PLC-γ1 (Null) or the same cells reconstituted with wild type PLC-γ1 (Plus) (Fig. 1*A*). When osmolality is raised from 300 to 500 mosmol/kg by adding NaCl, TonEBP/OREBP transcriptional activity increases 193-fold in Plus cells but only 25-fold in Null cells (Fig. 1*A*). As a control, using an otherwise identical luciferase reporter that does not contain a TonEBP/OREBP binding site (Fig. 1*A*, Promoter), transcriptional activity is not

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The authors declare no conflict of interest.

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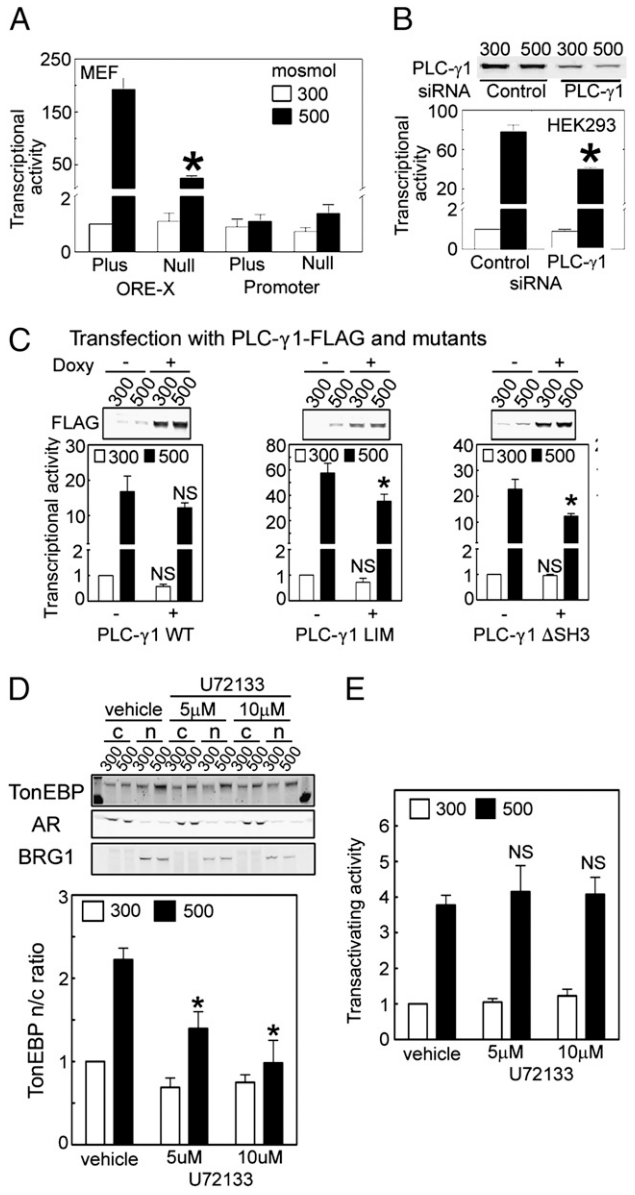

Fig. 1. PLC-γ1 contributes to the high NaCl–induced increase in TonEBP/OREBP transcriptional activity. After transfection, osmolality either was kept at 300 mosmol/kg or was increased to 500 mosmol/kg by adding NaCl for 16 h before measuring reporter activity. (*A*) TonEBP/OREBP transcriptional activity in MEFs deficient in PLC-γ1 (Null) and the same cells reconstituted with PLC-γ1 (Plus). Luciferase reporters are ORE-X and an otherwise identical reporter that does not contain a TonEBP/OREBP binding site, "Promoter." (*B*) Effect of siRNA knockdown of PLC-γ1 on TonEBP/OREBP transcriptional activity (ORE-X reporter) in cells transfected with control (nontargeting) or PLC-γ1-specific siRNA for 48 h. *Inset:* Effect of the siRNAs on PLC-γ1 protein expression. (*C*) Stimulation of TonEBP/OREBP by PLC-γ1 is dependent on its lipase activity. Effect of conditional (Tet-on) over expression of wild-type PLC-γ1 and dominant negative LIM PLC-γ1 in HEK293 cells. *Inset:* Expression of the Flag-PLC-γ1 constructs. (*D* and *E*) Effect of the phospholipase inhibitor U73122. U73122 or vehicle (DMSO) control was added from 30 min before changing osmolality. Mean ± SEM. $*P \leq 0.05$; $n = 3$.

affected by NaCl concentration and does not differ between Null and Plus cells. In addition, we knocked PLC-γ1 down in HEK293 cells with a specific siRNA. This siRNA reduces expression of PLC-γ1 protein by 80% at both 300 and 500 mosmol/kg (Fig. 1*B*, *Inset*). High NaCl increases TonEBP/OREBP transcriptional activity 78-fold in the presence of a "Control" nonspecific siRNA (Fig. 1*B*). In contrast, when PLC-γ1 is knocked down by the specific siRNA, TonEBP/OREBP transcriptional activity is reduced by 49% at 500 mosmol/kg without any significant effect at 300 mosmol/kg (Fig. 1*B*). To test whether this effect of PLC-γ1 depends on its lipase activity, we knocked down that activity by conditional (Tet-on) overexpression of a dominant negative lipase inactive mutant (LIM) of PLC-γ1 (7). In HEK293 cells overexpression of PLC-γ1-LIM-Flag reduces the high NaCl–induced increase of TonEBP/OREBP transcriptional activity (Fig. 1*C*). PLC-γ1-ΔSH3-Flag also reduces the high NaCl–induced increase of TonEBP/OREBP transcriptional activity (Fig. 1*C*). We conclude that PLC-γ1 contributes to the high NaCl–induced increase of TonEBP/OREBP transcriptional activity and that this effect involves both its lipase activity and its SH3 domain.

Lipase Activity of PLC-γ1 Contributes to the High NaCl–Induced Nuclear Localization of TonEBP/OREBP but Not to the Increase of Its Transactivating Activity. High NaCl activates TonEBP/OREBP by increasing its nuclear localization and transactivating activity (1). We inhibited PLC-γ1 phospholipase activity with U72133 (8) to test for possible effects on TonEBP/OREBP nuclear localization and transactivating activity. U72133 inhibits high NaCl–induced nuclear localization of TonEBP/OREBP (Fig. 1*D*) but not the high NaCl–induced increase of its transactivating activity (Fig. 1*E*).

High NaCl Increases Phosphorylation of TonEBP/OREBP at Y143. Because when Y143 in TonEBP/OREBP is phosphorylated it is a probable binding site for PLC-γ1, we tested for phosphorylation at this site by the use of protein mass spectrometry. We repeatedly detected ions characteristic of phosphorylation of TonEBP/OREBP at Y143 in nuclear extracts from HEK293 exposed for 2 h to 200 or 500 mosmol/kg (NaCl varied) (Fig. S1, Table S1). Interestingly, we found less than 1% as many characteristic ions in the cytoplasmic extracts.

We confirmed and quantified phosphorylation of TonEBP/OREBP-Y143 by use of a phosphospecific antibody. HEK293 cells were transfected either with TonEBP/OREBP-V5 (wild type) or with TonEBP/OREBP-Y143A-V5. At 300 mosmol/kg, whole-cell extracts immunoblotted with anti-V5 show strong signals from both constructs (Fig. 2*A*). In contrast, the anti-phospho-Y143 antibody produces a strong signal from wild-type TonEBP/OREBP-V5 but not from TonEBP/OREBP-Y143A-V5 (Fig. 2*A*). Furthermore, elevating NaCl significantly increases phosphorylation of wild-type TonEBP/OREBP-V5 at Y143 (Fig. 2*B*), and at 500 mosmol/kg the nuclear to cytoplasmic (n/c) ratio of TonEBP/OREBP-P-Y143 greatly exceeds the n/c ratio of wild-type TonEBP/OREBP-V5 (Fig. 2*C*). We conclude that TonEBP/OREBP is phosphorylated at Y143, that elevating NaCl increases the phosphorylation, and that the phosphorylated form is greater in the nucleus than in the cytoplasm.

PLC-γ1 Associates with TonEBP/OREBP, Dependent on Y143. We immunoprecipitated proteins from HEK293 cells that stably overexpress either TonEBP-1-547-V5 (the N terminus) or TonEBP-548-1531-Gal4 (the C terminus). Anti-V5 antibody coimmunoprecipitates TonEBP/OREBP-1-547 and PLC-γ1 regardless of medium osmolality (Fig. 3*A*). As a control, neither is immunoprecipitated by nonspecific IgG. Anti-Gal4 antibody immunoprecipitates TonEBP/OREBP-548-1531-Gal4 but not PLC-γ1 (Fig. 3*B*). Thus, PLC-γ1 associates with the N terminus of TonEBP/OREBP and not the C terminus. We next measured the association separately in nuclear and cytoplasmic extracts from HEK293 cells. Anti-V5 antibody coimmunoprecipitates TonEBP/OREBP-1-1531-V5 together with PLC-γ1 from both the nuclear and cytoplasmic protein extracts, regardless of medium osmolality (Fig. 3*C*). However, adding NaCl to 500 mosmol/kg increases the association 5-fold in the cytoplasm but

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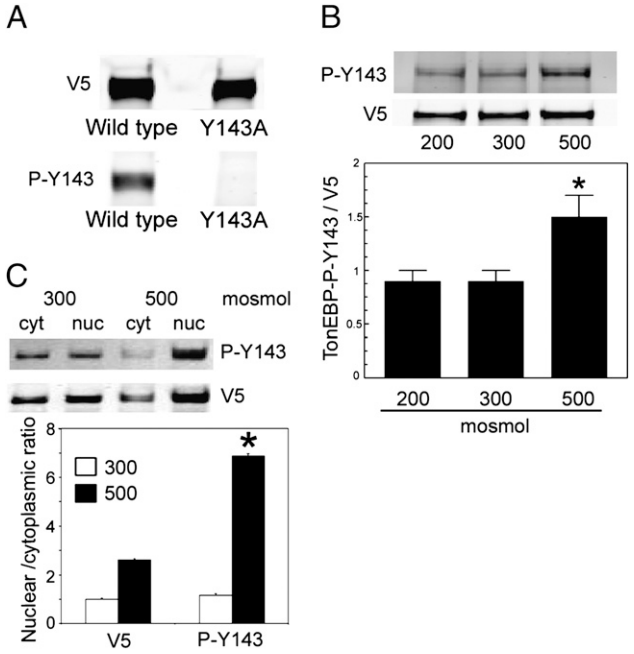

Fig. 2. High NaCl increases phosphorylation of TonEBP/OREBP at Y143. (*A*) Specificity of anti-phospho-Y143 TonEBP/OREBP antibody. HEK293 cells were transiently transfected with wild-type or mutated (Y143A) TonEBP/OREBP 1-1531-V5 at 300 mosmol/kg and exposed to 500 mosmol/kg for 1 h. (*B*) Effect of high NaCl on phosphorylation of TonEBP/OREBP at Y143. HEK293 cells transfected with wild-type TonEBP/OREBP-V5 were exposed to 200, 300, or 500 mosmol/kg (NaCl varied) for 1 h, and whole-cell extracts were examined by Western analysis. (*C*) High NaCl increases the n/c ratio of TonEBP/OREBP-P-Y143 more than it does the n/c ratio of wild-type TonEBP/OREBP-V5. As in *B*, except that nuclear and cytoplasmic proteins were extracted separately for analysis after 30 min. Mean ± SEM. $*P \leq 0.05$; $n = 3$.

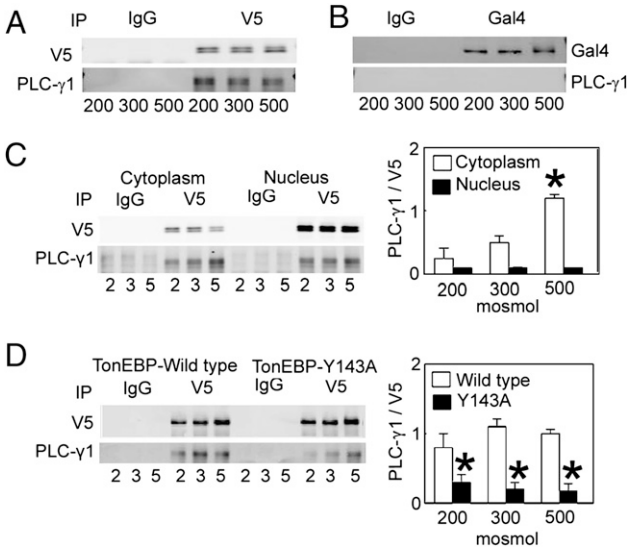

Fig. 3. PLC-γ1 physically associates with the N terminus of TonEBP/OREBP, dependent on Y143, but not with the C terminus. (*A*) PLC-γ1 coimmunoprecipitates with N-terminal TonEBP/OREBP. HEK293 cells stably expressing TonEBP/OREBP-1-547-V5 were grown at 300 mosmol/kg then exposed to 200, 300, or 500 mosmol/kg by varying NaCl for 2 h. IP with anti-V5 or IgG. (*B*) PLC-γ1 does not coimmunoprecipitate with C-terminal TonEBP/OREBP. As in *A*, except the cells stably express TonEBP/OREBP-Gal4dbd-548-1531. IP with anti-Gal4dbd or IgG. (*C*) NaCl-dependence of TonEBP/OREBP association with PLC-γ1 in cytoplasm and nucleus. As in *A*, except cells stably express TonEBP/OREBP-1-1531-V5. (*D*) The association depends on TonEBP/OREBP-Y143. As in *A*, except cells were transiently transfected with wild-type or mutated (Y143A) TonEBP/OREBP-1-1531-V5. Mean ± SEM. $*P \leq 0.05$; $n = 3$.

does not increase it in the nucleus (Fig. 3*C*). To determine whether TonEBP/OREBP Y143 is required for the association, we tested the effect of mutation of Y143 to alanine. Only 20% as much PLC-γ1 coimmunoprecipitates with transiently transfected TonEBP/OREBP-Y143A-V5 as with wild-type TonEBP/OREBP-V5, regardless of osmolality (Fig. 3*D*). The small amount of PLC-γ1 that coimmunoprecipitates with TonEBP/OREBP-Y143A-V5 could be associated with native TonEBP/OREBP that has formed a heterodimer with the TonEBP/OREBP-Y143A-V5. We conclude that PLC-γ1 physically associates, directly or indirectly, with TonEBP/OREBP at Y143 in its N terminus, and that high NaCl increases the association in the cytoplasm.

PLC-γ1 Is Part of the Protein Complex That Binds Along with TonEBP/OREBP to Its Cognate DNA Element, Osmotic Response Element. We performed electromobility shift assays using an osmotic response element (ORE) probe (9) and with or without antibody against PLC-γ1 (Fig. 4*A*). Addition of anti-TonEBP/OREBP to the binding reaction results in a supershift (Fig. 4*A*, lane 3). The TonEBP/OREBP-shifted band is eliminated by addition of 10-fold molar excess nonbiotinylated competitor, indicating that the mobility shift is specific (Fig. 4*A*, lane 1). Addition of anti-PLC-γ1 to the binding reaction reduces the signal from the shifted complex, presumably because binding of the antibody to PLC-γ1 interferes with formation of the complex (Fig. 4*A*, lane 5). The mean signal with antibody added is 40% ± 14%, $P < 0.05$ ($n = 3$), of the signal without antibody in flanking lanes. We conclude that PLC-γ1 is part of the protein complex associated with TonEBP/OREBP at its ORE binding sites.

PLC-γ1 Contributes to the High NaCl–Induced Increase of TonEBP/OREBP Transactivating Activity. High NaCl increases the transactivating activity of TonEBP/OREBP (10). We tested the effect of PLC-γ1 on this activity by use of a binary reporter system in PLC-γ1 Null and Plus MEF cells (Fig. 4*B*). High NaCl increases TonEBP/OREBP transactivating activity by 5-fold in Plus cells but by less than 2-fold in Null cells, and the activity at 500 mosmol/kg is significantly greater in Plus cells than in Null cells (Fig. 4*B*). Overexpression of PLC-γ1 with its SH3 domain deleted (ΔSH3) reduces TonEBP/OREBP transactivating activity at 500 mosmol/kg (Fig. 4*C*). Adding the deletion of its two SH2 domains has no further effect (Fig. 4*C*). We conclude that PLC-γ1contributes to the high NaCl–induced increase of TonEBP/OREBP transactivating activity and that its SH3 domain but not SH2 domains is necessary for this effect.

PLC-γ1 Contributes to High NaCl–Induced Nuclear Localization of TonEBP/OREBP, Dependent on TonEBP/OREBP-Y143. High NaCl–induced nuclear localization of TonEBP/OREBP is a factor in increasing its transcriptional activity. We measured the n/c ratio of TonEBP/OREBP by Western analysis in extracts from the cytoplasm and nucleus of PLC-γ1 Null and Plus cells (Fig. 5*A*). Increasing osmolality from 200 to 500 mosmol/kg by adding NaCl increases TonEBP/OREBP n/c ratio 20-fold in Plus cells but only 1.7-fold in Null cells, and the n/c ratio of TonEBP/OREBP at 500 mosmol/kg is significantly greater in Plus cells than in Null cells (Fig. 5*A*). We next tested the effect of mutation of TonEBP/OREBP Y143 to alanine on its n/c ratio in HEK293 cells (Fig. 5*B*). Increasing osmolality from 200 to 500 mosmol/kg causes a 5-fold increase of the n/c ratio of wild-type recombinant TonEBP/OREBP but only a 2.1-fold increase of TonEBP/OREBP-Y143A (Fig. 5*B*). The results are similar in MEF cells (Fig. 5*C*). In Plus cells, adding NaCl to 500 mosmol/kg increases wild-type TonEBP/OREBP n/c ratio by 4-fold but increases the

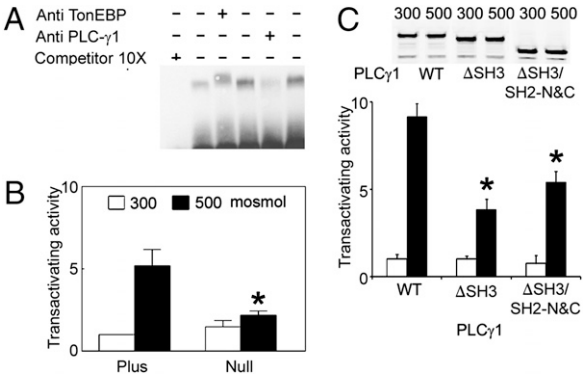

Fig. 4. (*A*) PLC-γ1 is part of the protein complex that associates with TonEBP/OREBP at its DNA binding sites. EMSA binding reactions were performed with nuclear extracts of HEK293 cells exposed to 500 mosmol/kg medium (NaCl added) for 2 h. The ORE probe (bp −1,238 to −1,104 of the human *AR* gene) contains three OREs. Additions to reactions include antibodies that recognize PLC-γ1 and TonEBP/OREBP or 10-fold excess of nonbiotinylated probe (representative of three independent experiments). (*B* and *C*) PLC-γ1 contributes to high NaCl–dependent TonEBP/OREBP transactivating activity. (*B*) PLC-γ1 Null and Plus cells were cotransfected with pFR-Luc and TonEBP/OREBP C-terminal GAL4dbd-548-1531. After 24 h, osmolality either was kept at 300 mosmol/kg or was increased to 500 mosmol/kg for 16 h by adding NaCl. (*C*) HEK293 cells were cotransfected with pFR-Luc and TonEBP/OREBP C-terminal GAL4dbd-548-1531 and with either wild-type PLC-γ1 (WT), PLC-γ1 in which the SH3 domain was deleted (ΔSH3), or with PLC-γ1 in which the SH3 and both SH2 domains were deleted (ΔSH3/SH2-N&C). After 24 h, osmolality either was kept at 300 mosmol/kg or was increased to 500 mosmol/kg for 16 h by adding NaCl. Mean ± SEM. $*P \leq 0.05$; $n = 3$.

n/c ratio of TonEBP/OREBP-Y143A only by 2-fold. In the Null cells adding NaCl to 500 mosmol/kg does not increase n/c ratio of either wild-type or Y143A-mutated TonEBP/OREBP. In addition, we compared the effect on TonEBP/OREBP n/c ratio in MEFs of mutating either of the two SH2 domains in PLC-γ1 (Fig. 5*D*). In Null cells, adding NaCl to 500 mosmol/kg increases the n/c ratio of TonEBP/OREBP more than 4-fold when either wild-type PLC-γ1 or PLC-γ1 with mutated SH2-N domain is overexpressed, but there is no significant increase in the ratio if the SH2-C domain is mutated. We conclude that PLC-γ1 contributes to TonEBP/OREBP nuclear localization at elevated NaCl through interaction between TonEBP/OREBP Y143 and the PLC-γ1 SH2-C binding domain. Transfecting PLC-γ1 null cells with wild-type PLC-γ1 increases TonEBP/OREBP n/c ratio at 500 mosmol/kg (Fig. 5*E*). The result is the same when the PLC-γ1 SH3 domain is deleted, indicating that domain is not necessary for the effect (Fig. 5*E*).

High NaCl Increases the Nuclear Localization of PLC-γ1. We measured the n/c ratio of PLC-γ1 in HEK293 cells (Fig. 5*F*). PLC-γ1 is mainly located in the cytoplasm, but increasing osmolality from 200 to 500 mosmol/kg by adding NaCl causes a 3-fold increase in its n/c ratio.

Discussion

We find that PLC-γ1 contributes to high NaCl–induced increase of the transcriptional activity of TonEBP/OREBP (Fig. 1 *A* and *B*) by increasing its transactivating activity (Fig. 4*B*) and by increasing its nuclear localization (Fig. 5). PLC-γ1 plays a central role in signal transduction by cleaving phosphatidyl inositol into diacylglycerol and the second messenger inositol triphosphate (11). PLC-γ1 lipase activity contributes to high NaCl–induced activation of TonEBP/OREBP (Fig. 1*C*) by increasing its nuclear localization (Fig. 1*D*) but not its transactivating activity (Fig. 1*E*). It can also signal independently of its lipase activity. For example, PLC-γ1 is required for agonist-induced Ca^{2+} entry. Both wild-type and lipase-inactive PLC-γ1 are effective, but an SH3 domain deletion mutant is not (12). A probable mechanism is that PLC-γ1 can act as a guanine nucleotide exchange factor (GEF), independent of its lipase activity. For example, it acts through its SH3 domain as a GEF for the GTPase dynamin-1 in mediation of clathrin-dependent endocytosis of the EGF receptor (13). Additionally, its GEF activity can activate phosphatidylinositol 3-kinase (PI3K-1A). For example, NGF triggers localization of PLC-γ1 to the nucleus, where it acts as a GEF for the GTPase, phosphatidylinositol-3-kinase enhancer (PIKE), and PIKE, in turn, activates nuclear PI3K (14, 15). This signaling cascade may contribute to high NaCl–induced activation of TonEBP/OREBP because deletion of the PLC-γ1 SH3 domain (Fig. 4*C*) and inhibition of PI3K-1A (16) both reduce that activation.

PLC-γ1 is mainly cytoplasmic, but some of it is in the nucleus (17–19) and NGF increases its nuclear localization (15). Likewise, high NaCl also increases nuclear localization of PLC-γ1 (Fig. 5*F*). High NaCl increases phosphorylation of TonEBP/OREBP on Y143 (Fig. 2*B*). The binding of PLC-γ1 SH2-C to other proteins depends on such phosphorylation of tyrosine (11). PLC-γ1 associates with TonEBP/OREBP in the cytoplasm, and this association increases with high NaCl (Fig. 3*C*), provided that Y143 is available for phosphorylation (Fig. 3*D*). Lack of either Y143 or PLC-γ1 reduces high NaCl–induced accumulation of TonEBP/OREBP in the nucleus (Fig. 5 *A*–*C*). We propose that high NaCl causes phosphorylation of TonEBP/OREBP Y143 in the cytoplasm; that PLC-γ1 binds to TonEBP/OREBP-Y143 through its SH2-C domain; and that TonEBP/OREBP-P-Y143 together with PLC-γ1 then accumulate in the nucleus (Figs. 2*C* and 5*F*), where they remain associated (Fig. 3*C*). The amino acid sequence in which TonEBP/OREBP-Y143 is located makes it a probable site for c-Abl kinase and SHP-1 phosphatase (Minimotif Miner; http://mnm.engr.uconn.edu/MNM/SMSSearch-Servlet), making them candidates for mediation of high NaCl–induced phosphorylation of TonEBP/OREBP at Y143.

In the nucleus, TonEBP/OREBP binds to its cognate DNA elements (OREs) in association with PLC-γ1 (Fig. 4*A*). Association of PLC-γ1 with TonEBP/OREBP at OREs may be involved in the high NaCl–induced increase of transactivating activity of TonEBP/OREBP (Fig. 4*B*). The binary transactivation assay that was used contains TonEBP/OREBP C-terminal amino acids 548–1,531, which include its tonicity-dependent transactivation domain (TAD) (10). PLC-γ1 apparently does not associate directly with this region of TonEBP/OREBP (Fig. 3*B*), but it could act through an intermediary TonEBP/OREBP partner such as ATM or PI3K-1A, which associate with TonEBP/OREBP to stimulate its transactivating activity (16, 20). PI3K-1A interacts with both ATM (16) and PLC-γ1 (14, 15). The relationship between PLC-γ1 and PI3K-1A could be important in this context because in other systems, where PLC-γ1 is stimulated to enter the nucleus as it is with high NaCl (Fig. 5*F*), PLC-γ1 activates PI3K-1A through PIKE (14, 15).

Interaction of PLC-γ1 with TonEBP/OREBP phosphorylated on Y143 contributes to high NaCl–induced nuclear localization of the TonEBP/OREBP (Fig. 5). Y143 is in a region (amino acids 132–156) previously designated as an auxiliary export domain (AED), necessary for export of TonEBP/OREBP from the nucleus under conditions of normotonicity or hypotonicity (21). Deletion of the AED was reported to result in nuclear localization of TonEBP/OREBP regardless of tonicity (21). In HeLa cells TonEBP/OREBP-132-581-FLAG is nuclear at 300 mosmol/kg and cytoplasmic at 260 mosmol/kg (22). Mutation of S155 to alanine prevents this hypotonicity-induced export of TonEBP/OREBP from the nucleus, but mutation of Y143 to alanine does not (22). The effect of hypertonicity on the export was not reported. Possibly, high NaCl–induced phosphorylation of Y143 and subsequent interaction with PLC-γ1 might reduce flux of TonEBP/OREBP out of the nucleus under that specific

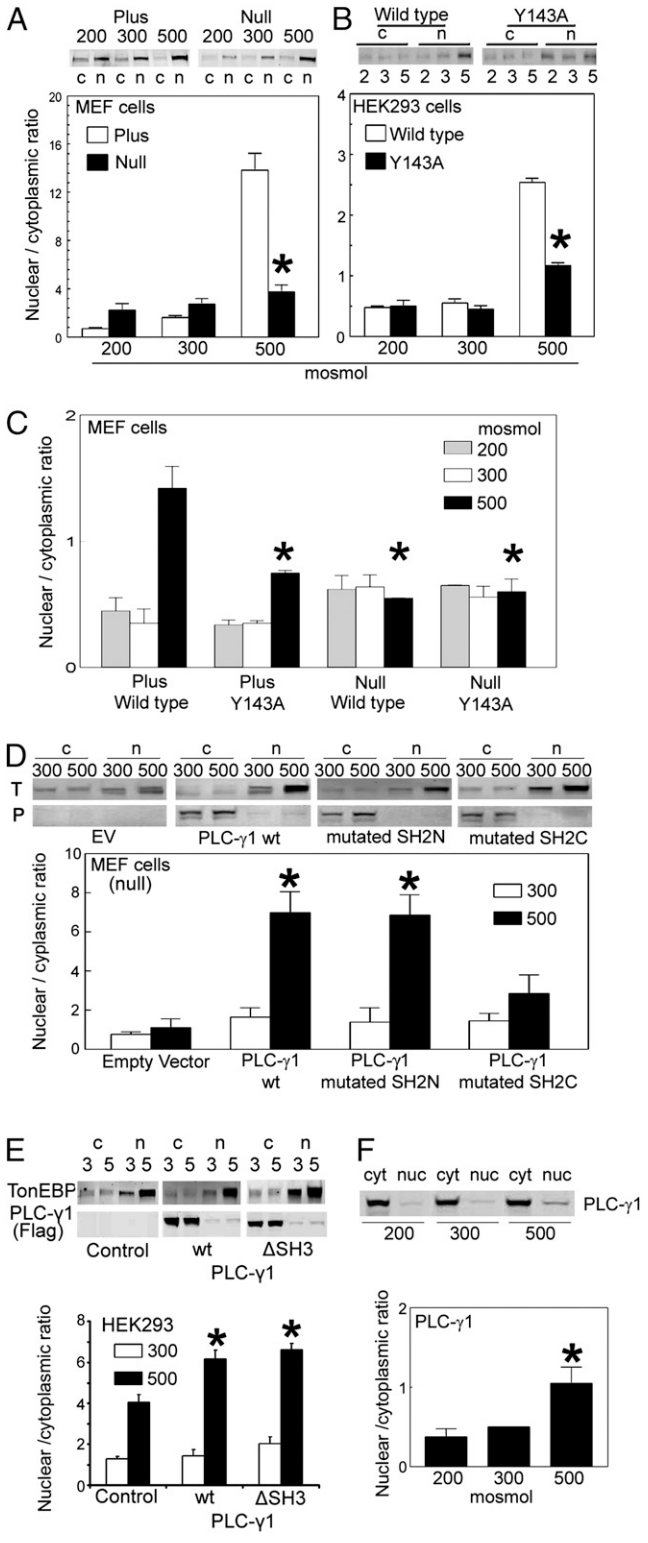

Fig. 5. Cells were exposed to 200, 300, or 500 mosmol/kg by varying NaCl for 30 min before extracting nuclear and cytoplasmic proteins for Western analysis. (*A*) PLC-γ1 contributes to high NaCl–dependent nuclear localization of TonEBP/OREBP. Native TonEBP/OREBP n/c distribution in MEF deficient in PLC-γ1 (Null) and the same cells reconstituted with PLC-γ1 (Plus). (*B*) High NaCl–induced nuclear localization of TonEBP/OREBP depends on TonEBP/OREBP-Y143. As in *A*, except that HEK293 cells were transfected with wild-type or mutated (Y143A) TonEBP/OREBP-1-1531-V5. Anti-V5 antibody was used. (*C*) Mutation of TonEBP/OREBP-Y143 and absence of PLC-γ1 have equivalent and nonadditive inhibitory effects on high NaCl–induced nuclear localization of TonEBP/OREBP. Null and Plus MEF were transfected as in *B*. (*D*) The SH2-C domain of PLC-γ1 is necessary for its contribution to high NaCl–induced nuclear localization of TonEBP/OREBP. The n/c ratio of native TonEBP/OREBP was measured in Null cells transiently transfected with wild-type PLC-γ1 or PLC-γ1 mutated at its SH2-N or SH2-C domain. Immunoblots: T, anti-TonEBP; P, anti- PLC-γ1. (*E*) HEK293 cells not induced (Control) or conditionally induced (Tet-on) to overexpress wild-type or SH3 deleted (ΔSH3) PLC-γ1. Wild-type and ΔSH3 increase native TonEBP/OREBP n/c ratio equally at 500 mosmol/kg. (*F*) High NaCl increases the n/c ratio of PLC-γ1. Mean ± SEM. $*P \leq 0.05$; $n = 3$.

condition, thus increasing its nuclear localization. However, additional studies are necessary to distinguish between this and other possibilities.

Experimental Procedures

Cell Culture and Treatment. HEK293 were grown as previously described (20) and HEK293 Tet-on cells according to the supplier's recommendations (Clontech). Flag PLC-γ1 constructs were transiently transfected into cells with doxycycline present (PLC-γ1 constructs expressed). MEFs genetically deficient in PLC-γ1 (Null cells) and retrovirally infected to reexpress PLC-γ1 (Plus cells) (23) were kindly provided by Dr. Graham Carpenter, Vanderbilt University. At experiment-specific time points, medium was replaced with ones at 300 mosmol/kg, 200 mosmol/kg (NaCl added to NaCl-free medium; Biofluids), or 500 mosmol/kg (NaCl added). The phospholipase C inhibitor U72133 (8) was from Sigma Aldrich. It was added to medium bathing HEK293 cells 30 min before changing the osmolality.

Plasmids and siRNA. The ORE-X luciferase reporter of TonEBP/OREBP transcriptional activity contains two copies of the human TonEBP/OREBP DNA binding element ORE-X (24) within a minimal IL-2 promoter (25) upstream of the *Photinus pyralis* luciferase gene (hTonE-GL3, kindly provided by Dr. Steffan N. Ho, University of California, San Diego), as previously described (20).

Constructs containing TonEBP/OREBP amino acids 1–547 or 1–1,531 were described previously (20). The mutant Y143A of 1-1531-V5 was prepared by site-directed mutagenesis (Quik Change; Stratagene).

The binary GAL4 reporter system was previously described (10).

Rat PLC-γ1 wild type, SH2-N mutant (R586A), and SH2-C mutant (R694A, R696A) (26) were from Dr. Sue Goo Rhee, Ewha Womans University, Seoul, Korea. Conditional expression vectors for rat PLC-γ1 wild type, LIM (H335Q), and SH3 deletion mutants (ΔSH3) (13) were provided by Dr. Pann-Ghill Suh, Pohang University of Science and Technology, Pohang, Korea.

The siRNA against PLC-γ1 (27) was redesigned as a synthetic dsRNA Dicer substrate to enhance RNA interference potency and efficacy (28). The siRNA sequences were as follows: sense, 5′-Phos-AAGAAGUCGCAGCGACCCGAGdCdG-3′ and antisense, 5′-CGCUCGGGUCGCUGCGACUUCUU-3′. The control (nontargeting) siRNA (Integrated DNA Technologies) sequences were as follows: sense, 5′-Phos-UGAACCUGACCCAGGGGAGGGAGdTdT-3′, and antisense, 5′-AACUCCCUCCCC UGGGUCAGGUUCAUU-3′ (16).

Transfection and Luciferase Assays. Cells were transfected with Lipofectamine 2000 (Invitrogen), according to the supplier's instructions. For assay of the transcriptional activity of native TonEBP/OREBP, cells were transfected with ORE-X or promoter only (control) reporters (20). For assay of the activity of the TonEBP/OREBP TAD, cells were cotransfected with the GAL4 reporter (pFR-Luc) and GAL4dbd-548-1531, which contains the recombinant TAD (10). For siRNA, cells stably expressing the ORE-X reporter were transfected with 20 nM siRNA.

EMSA was as previously described (9).

The IP procedure was as previously described (20).

Liquid Chromatography–Multiple Reaction Monitoring–Mass Spectrometry. Dynabeads (Dynal Biotech) containing immunoprecipitated TonEBP/OREBP 1-547-V5 were resuspended in 6 M guanidine-HCl/50 mM NH_4HCO_3, to denature and elute the protein. The protein was reduced and alkylated, then the buffer was exchanged to 100 mM Tris-HCl, 10 mM $CaCl_2$, pH 7.6, for digestion with endoproteinase Arg-C (Roche Applied Science) in a ratio of 1:50 wt/wt overnight at 37°C. Protein digests were reconstituted in 20 μL of 5% acetonitrile (ACN) and 0.2% formic acid, then analyzed by nano–liquid chromatography–multiple reaction monitoring–mass spectrometry (LC-MRM-MS) on a 4000 Q TRAP hybrid triple quadrupole/linear ion trap mass spectrometer (Applied Biosystems|;MDS SCIEX). Chromatography was carried out using the TEMPO MDLC system using nanoflow composed of solvent A (2% ACN, 0.1% formic acid) and solvent B (98% ACN, 0.1% formic acid). Three to four MRM

transitions were monitored and acquired at unit resolution for each peptide of interest. Simultaneous collection of MS/MS data were carried out to sequence and validate the targeted peptides.

Western blots were performed as previously described (16), using rabbit anti-NFAT5 (TonEBP/OREBP) (Affinity BioReagents), mouse anti-Flag (Sigma-Aldrich), rabbit anti-PLC-γ1 (Cell Signaling), mouse anti-V5 (Invitrogen), mouse anti-Gal4dbd (Santa Cruz Biotechnology), or rabbit anti-TonEBP/OREBP phospho-Y143 (Phospho-Solutions, Aurora, CO).

Calculation of n/c Ratios. The relative amounts of TonEBP/OREBP in the cytoplasmic and nuclear fractions and the n/c ratio were calculated from the relative concentrations of TonEBP/OREBP in each cytoplasmic or nuclear extract and the relative volumes of the extracts (9).

Statistical Analysis. Data were compared by a repeated-measures ANOVA followed by Bonferroni multiple comparison test for separation of significant means. Normalized data were log-transformed before ANOVA. Differences were considered significant at $P \leq 0.05$.

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