Environmental hyperosmolality regulates phospholipid biosynthesis in the renal epithelial cell line MDCK

Cecilia I. Casali, Karen Weber, Nicolás O. Favale, and María C. Fernández Tome[1]

Department of Biological Sciences, School of Pharmacy and Biochemistry, University of Buenos Aires, IQUIFIB–CONICET, Ciudad Autónoma de Buenos Aires (C1113AAD), Argentina

Abstract **Hyperosmolality is a key signal for renal physiology. On the one hand, it contributes to the differentiation of renal medullary structures and to the development of the urinary concentrating mechanism. On the other, it is a stress factor. In both cases, hyperosmolality activates processes that require an adequate extension of cellular membranes. In the present work, we examined whether hyperosmolality regulates phospholipid biosynthesis, which is needed for the membrane biogenesis in the renal epithelial cell line Madin-Darby canine kidney (MDCK). Because phospholipids are the structural determinants of all cell membranes, we evaluated their content, synthesis, and regulation in MDCK cultures subjected to different hyperosmotic concentrations of NaCl, urea, or both. Hyperosmolality increased phospholipid content in a concentration-dependent manner. Such an effect was exclusively due to changes in NaCl concentration and occurred at the initial stage of hyperosmolar treatment concomitantly with the expression of the osmoprotective protein COX-2. The hypertonic upregulation of phosphatidylcholine (PC) synthesis, the main constituent of all cell membranes, involved the transcriptional activation of two main regulatory enzymes, choline kinase (CK) and cytidylyltransferase α (CCTα) and required ERK1/2 activation. Considering that physiologically, renal medullary cells are constantly exposed to high and variable NaCl, these findings could contribute to explaining how renal cells could maintain cellular integrity even in a nonfavorable environment.**—Casali, C. I., K. Weber, N. O. Favale, and M. C. F. Tome. **Environmental hyperosmolality regulates phospholipid biosynthesis in the renal epithelial cell line MDCK. *J. Lipid Res.* 2013.** 54: **677–691.**

Supplementary key words osmotic stress • membrane biogenesis • renal epithelial cells • Madin-Darby canine kidney

Hyperosmolality is a key signal for renal physiology (1, 2). On the one hand, high renal medullary interstitial osmolality contributes to the differentiation of tubular structures and to the maturation of the urinary concentrating system in the developing kidney (3). In addition, hyperosmolality is an absolute requirement for proper urine concentration in the mature kidney (3, 4). On the other hand, hyperosmolality is considered a stress factor. The high and variable concentrations of sodium and urea in renal interstitial tissue can severely affect the structure and function of renal cells. Therefore, to live in such an adverse physiological environment, cells have to implement numerous compensatory and defensive mechanisms that include the expression of various osmoprotective proteins such as the molecular chaperone Hsp70 (5), the membrane cotransporters sodium/myo-inositol (SMIT), sodium/chloride/betaine (BGT1), sodium/chloride/taurine (TauT) (6–9), and the enzyme cyclooxygenase 2 (COX2) (10–12), among others. All of these proteins are transcriptionally activated by the tonicity-responsive enhancer binding protein (TonEBP), which is thought to be the master regulator of tonicity changes in renal tissue (13–15). All of the cellular processes mentioned above have an absolute requirement of cellular membranes. The polarization differentiation process comprises the increase in cell size and volume and, in consequence, the extent of cellular membranes (16–20). In addition, hyperosmolality induces the expression of a large number of osmoprotective genes whose expression renders membrane-bound proteins; moreover, their biosynthesis is a membrane-attached process, inasmuch as it occurs at endoplasmic reticulum and Golgi apparatus membranes (21). Thus, it is logical to suppose that environmental osmolality could regulate the generation of new membranes

Membrane biogenesis implies the coordinated synthesis and assembly of all membrane components (21, 22), i.e., proteins and lipids; but the basic structural feature of all mammalian cell membranes is the phospholipid bilayer (23). Thus, the biosynthesis of phospholipid can be considered a parameter of cell membrane biogenesis. Previous work

This work was supported by the University of Buenos Aires, UBACyT 20020090200251 and CONICET, PIP 0327 grants.

Manuscript received 24 August 2012 and in revised form 14 December 2012.

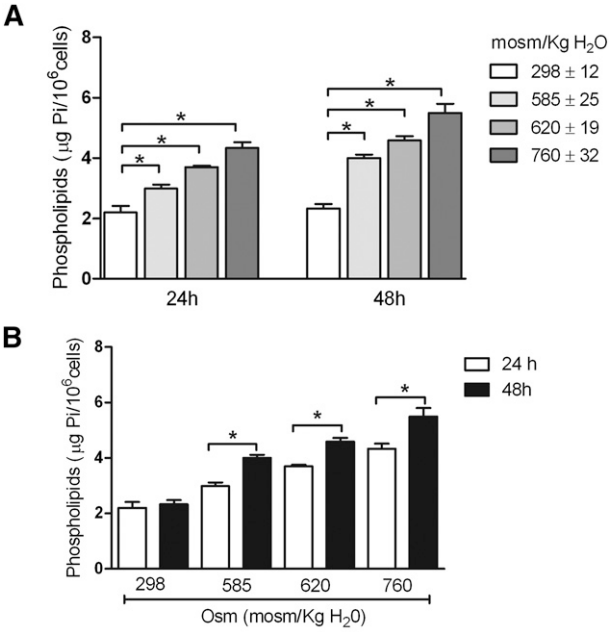
Published, JLR Papers in Press, December 26, 2012
DOI 10.1194/jlr.M031500

Abbreviations: CCTα, cytidylyltransferase α; CK, choline kinase; CPT, choline phosphotransferase; DAG, diacylglycerol; GPC, glycerophosphocholine; MDCK, Madin-Darby canine kidney; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PLC, phospholipase C; PLD, phospholipase D; PS, phosphatidylserine.

[1]To whom correspondence should be addressed.
e-mail: fertome@ffyb.uba.ar

from our laboratory demonstrated that the inner renal medulla has the most-active phospholipid synthesis and membrane turnover of all kidney zones (24). We have demonstrated that the ability of the inner renal medulla to renew the phospholipidic constituents of cell membranes is a defense mechanism against diverse injuries (25–27). We have speculated that such an active phospholipid metabolism in renal medullary cells is a consequence of the high interstitial osmolality, but we did not confirm this hypothesis. Madin-Darby canine kidney (MDCK) is a cell line that has been typified as a transporting-polarized epithelium that possesses both morphological and enzymatic properties of cells from distal tubules and medullary collecting ducts of the kidney (28, 29). Recently, it has been reported that cultures of MDCK subjected to osmotic stress were induced to synthesize and accumulate the sulfoglycosphingolipids SM4 and SM3 that are constituents of renal membranes (30, 31). It was also shown that sulfoglycosphingolipid accretion increased along with the osmolality of the incubation medium, which also increased the mRNA of biosynthetic enzymes. In addition, it has been demonstrated that hypertonicity induces MDCK cell differentiation by regulating glycosphingolipid synthesis and accumulation in the apical membrane of MDCK cells (32). However, whether hyperosmolality affects phospholipid content and biosynthesis has not yet been determined.

In the present work, we explored whether hyperosmolality regulates membrane phospholipid content, composition, and biosynthesis. For this purpose, we evaluated phospholipid metabolism in cultures of the renal epithelial cell line MDCK subjected to hyperosmotic concentrations of the main renal interstitial solutes, NaCl, and/or urea. We also explored some mechanisms on how hypertonic environment regulates this metabolic process and the possible participation of the osmotic transcriptional regulator TonEBP/NFAT5.



Medium	+	+	+	+
NaCl (mM)	-	100	125	150
Urea (mM)	-	100	100	200
Osm (mosm/Kg H_2O)	298 ± 12	585 ± 25	620 ± 19	760 ± 32

Fig. 1. Hyperosmolality increases membrane phospholipid content. MDCK cells were grown in a mixture containing DMEM/Ham's-F12 (1:1), 10% FBS, and 1% antibiotic mixture. After reaching 70–80% confluence, cells were placed in low-serum medium (0.5% FBS) for 24 h and then subjected to hyperosmolality for 24 h and 48 h. Hyperosmolar media were made by adding aliquots of sterile 5 M NaCl and 10 M urea to commercial medium to achieve the desired final osmolalities according to the table at the bottom of Fig. 1. After treatment, cells were collected and lipids analyzed as described in Materials and Methods. A: Total phospholipid content as a function of the medium osmolality. Results express the mean ± SEM of five independent experiments. *Significantly different from control values (298 mosm/kg H_2O), $P < 0.05$. B: Total phospholipid content as a function of incubation time. Results express the mean ± SEM of five independent experiments. *Significantly different from 24 h values at each osmolality assayed, $P < 0.05$.

MATERIALS AND METHODS

Cell culture conditions

MDCK cells (American Type Culture Collection, passages 45–50) were grown in a mixture containing DMEM and Ham's-F12 (1:1), 10% FBS, and 1% antibiotic mixture (GIBCO®). After reaching 70–80% confluence, cells were placed in low-serum medium (0.5% FBS) for 24 h and then subjected to hyperosmolality for different periods of time. Hyperosmolar media were made by adding aliquots of sterile 5 M NaCl or 10 M urea or both, NaCl and urea, to commercial medium to achieve desired final osmolalities (see tables in **Figs. 1, 2**) that were determined by using an osmometer (μOSMETTE, Precision Systems; Sudsbury, MA). It is worth mentioning that no changes in final osmolalities were found in the media due to the different serum concentration.

After the treatments, the culture medium that contained dead cells and debris was discarded, and cells were washed twice with sterile PBS and treated with 0.25% trypsin-EDTA (GIBCO®) for 3 min. When cells were detached from the culture support, 20% of FBS was added to stop trypsin action. Cells were counted in a hemocytometer chamber (Neubauer´s chamber) in the presence of trypan blue to obtain the number of total and viable cells. Viability was calculated from these data as the percentage of non-trypan blue-stained cells of total counted cells.

Aliquots of cell suspensions containing an adequate number of cells were used for the different experimental protocols. Although nonviable cells were included in the cell population used in the experiments, the number of trypan blue-stained cells was lower than 10% (data included in Fig. 2C).

When it was required, cells were resuspended in lysis buffer (0.089% NaCl-phosphate buffer, pH 7.2, containing 0.05% Triton X-100, 1 mM PMSF, 10 μg/ml aprotinin, 1 mM leupeptin, and 1 mM sodium othovanadate). Protein determination in the lysates was carried out using the Lowry procedure (33).

Flow cytometry

To determine the effect of hyperosmolality on the cell cycle, DNA cell cycle analysis was measured on 70% ethanol-fixed, propidium iodide-stained cells according to the protocol previously described (34). In brief, MDCK cells were treated and harvested as described in cell culture conditions. Then, MDCK cell pellets were resuspended in 1 ml of ice-cold PBS, centrifuged, fixed by adding ice-cold 70% ethanol, and stored at −20°C. For propidium iodide staining, the fixed cells were washed once with PBS and

Medium	NaCl	Osm	Urea	Osm	NaU		Osm
					NaCl	Urea	
Iso	-	298 ± 12	-	298 ± 12	-	-	298 ± 12
A	100	475 ± 18	75	375 ± 16	100	100	585 ± 25
B	125	512 ± 12	100	406 ± 17	125	100	620 ± 19
C	150	580 ± 21	200	506 ± 22	150	200	760 ± 32

Osm (mosm/Kg H_2O); *Na Cl* (mM); *Urea* (mM)

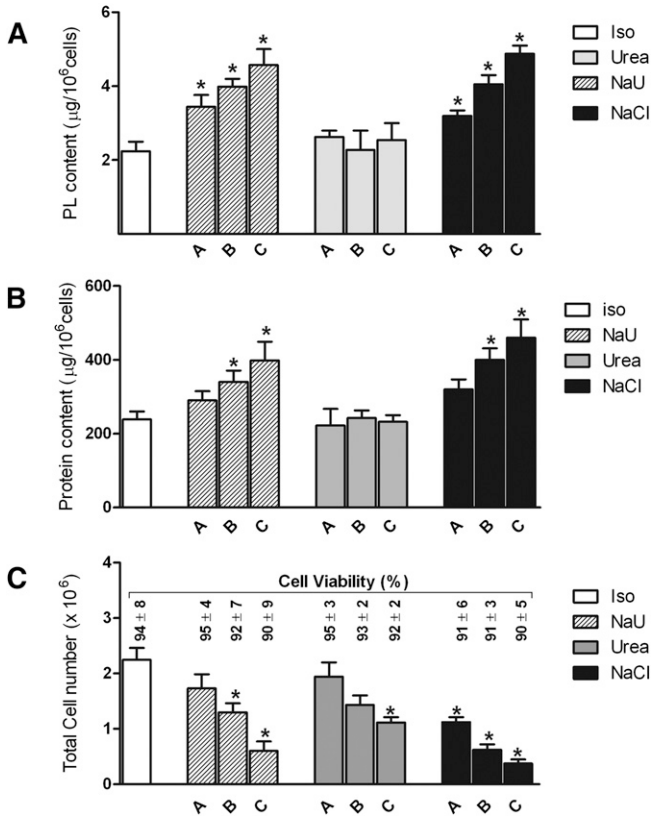


Fig. 2. High NaCl, but not high urea, regulates phospholipid mass increase. MDCK cells were grown in a mixture containing DMEM/Ham's-F12 (1:1), 10% FBS, and 1% antibiotic mixture. After reaching 70–80% confluence, cells were placed in low-serum medium (0.5% FBS) for 24 h and then subjected to different concentrations of NaCl, urea, or both for 24 h. Hyperosmolar media were made by adding aliquots of sterile 5 M NaCl or 10 M urea or both (NaU) to commercial medium to achieve the desired final osmolalities according to the table at the top of the figure. After treatment, cells were collected and then used for phospholipid mass determination (A), protein mass determination (B), and cell number counting and viability determinations (C). Results express the mean ± SEM of five independent experiments. *Significantly different from isosmolar control values (Iso, 298 mosm/kg H_2O), $P < 0.05$.

incubated in the presence of PI (50 µg/ml), 0.1% Triton X-100, and 50 µg/ml RNase A in PBS for 30 min at 37°C. Analysis was performed with a flow cytometer (FACSCalibur™; Becton Dickinson). After cell doublets exclusion with WinMDI version 2.9 (The Scripps Research Institute; La Jolla, CA) cell cycle analysis of DNA histograms of 10,000 events was performed using Cylchred (Cardiff University, UK).

Lipid extraction

Total lipids were extracted by the method of Bligh and Dyer (35). Briefly, in the first step, MDCK cell pellets (~4×10^6 cells) were resuspended in 800 µl of PBS and mixed with 2 ml of methanol and 1 ml of chloroform, vortexed gently for 30 s, and incubated on ice for 15 min. To yield two phases, in a second step, 1 ml of chloroform and 1 ml of water were added to the samples, then vortexed for 30 s and centrifuged at 800 *g* for 5 min. The lower organic phase containing total cellular lipids was collected, dried under a nitrogen stream, and kept at −80°C for further analysis.

Phospholipid separation and quantification

Phospholipid species were separated by TLC using a two-solvent system (24). First, dried extracts containing total lipids were resuspended in 40 µl of chloroform and applied drop by drop onto a 1 cm lane of thin-layer silica gel chromathoplates. Then, plates were developed in the first solvent mix containing chloroform-methanol-acetic acid-water (40:10:10:1, v/v), dried, and developed in the second solvent mix containing chloroform-methanol-acetic acid-water (120:46:19:3, v/v). After chromatography, plates were exposed to iodine vapors to reveal phospholipid spots. Phospholipids were identified by comparison with the corresponding standards and the retention factors (Rfs): 0.20, 0.30, 0.47, 0.55, and 0.70 for sphingomyelin, phosphatidylcholine, phosphatidylinositol, phosphatidylserine, and phosphatidylethanolamine, respectively (24). The quantification of the different phospholipids was carried out by measuring the quantity of free orthophosphate according to the Bartlett procedure (36). Briefly, the zones of the plates containing phospholipid mass were scraped into a Kjeldhal tube, mixed with 600 µl of 70% perchloric acid and one drop of 0.5% ammonium molybdate, and heated at 200°C for 30 min to complete mineralization. This procedure allows the release of the phospholipid polar head-bound phosphate. The concentration of free orthophosphate was determined by using Fiske-Subarrow reagent (36).

Labeling experiments

To evaluate whether hyperosmolality affects membrane biogenesis, we studied phospholipid biosynthesis as a parameter of membrane biogenesis. The generation of new phospholipid molecules was evaluated by incorporating radioactive glycerol into phospholipids. Cells were grown to 70–80% confluence, placed in low-serum medium (0.5% FBS) for 24 h, and then subjected to hyperosmolality (125 mM NaCl and/or 100 mM urea) for different periods of time (3, 6, 9, 12, 24, 48, and 72 h). Three hours before cell harvesting, 2 µCi/ml of [U-^{14}C]glycerol (PerkinElmer®) was added to each well. In another set of experiments, phospholipid synthesis was assessed by measuring the incorporation of [^{32}P]orthophosphate into phospholipid molecules. For this purpose, cells were grown to 70–80% confluence, placed in low-serum medium (0.5% FBS) for 24 h, and then subjected to hyperosmolality (125 mM NaCl) for different periods of time (3 h, 24 h). Three hours before cell harvesting, 1 µCi/ml of [^{32}P]orthophosphate carrier-free (PerkinElmer®) was added to each well.

To determine which signaling pathway was involved in the activation of the phospholipid biosynthetic process, specific inhibitors were added 30 min before the addition of NaCl to the medium. The participation of phospholipase C (PLC) was evaluated by using 1 µM U73122 (Calbiochem). Phospholipase D (PLD) action was evidenced by using 0.1% buthanol (Merck), 0.15 µM VU0359595 (Avanti Polar Lipids) PLD1 inhibitor, and 0.5 µM VU0285655-1 (Avanti Polar Lipids) PLD2 inhibitor. To evaluate ERK1/2, 0.1 µM U0126 (Sigma-Aldrich) was used. After treatment with inhibitors, 125 mM of NaCl was added, and cells were cultured for 3 h and 24 h. As mentioned above, 3 h before cell harvesting, 1 µCi/ml of [^{32}P]orthophosphate carrier-free (PerkinElmer®) was added to each well. After labeling, cells were collected and counted as described above and then subjected to lipid extraction. Phospholipidic species were separated by TLC, and the radioactivity incorporated into each phospholipid was

visualized by radioautography and quantified by liquid scintillation counting.

Western blot analysis

After the correspondent treatments, cells were collected and counted as described above and suspended in lysis buffer (0.089% NaCl-phosphate buffer, pH 7.2, containing 0.05% Triton X-100, 1 mM PMSF, 10 μg/ml aprotinin, 1 mM leupeptin, and 1 mM sodium othovanadate). Aliquots of 200,000 cells were incubated with 4× Laemmli buffer at 100°C for 5 min, resolved in a 10% SDS-polyacrylamide gel, and blotted to polyvinylidene difluoride membranes (GE Healthcare). Blots were blocked with 10% nonfat milk in TBS-Tween and incubated overnight at 4°C with primary antibodies: rabbit polyclonal COX-2 antibody, 1:250 (Cayman Chemical Co.), rabbit polyclonal NFAT5 antibody, 1:200 (Santa Cruz Biotechnology), rabbit polyclonal P-ERK1/2 and total ERK antibodies, 1:3,000 (Cell Signaling), and rabbit polyclonal β-tubulin antibody, 1:5,000 (AbCam). After washing, blots were incubated with secondary antibody: goat anti-rabbit HRP conjugate 1:6,000 (GE Healthcare Lifescience), and bands were evidenced by means of ECLPlus Western blotting analysis system (GE Healthcare Lifescience). The intensity of each band was estimated by optical densitometry with Gel-Pro Analyzer 3.1.

RT-PCR

MDCK cells were grown, treated, and collected as described above, then 2×10^6 cells were used for total RNA extraction by using the SV Total RNA Isolation System (Promega) in accordance with the manufacturer's instructions. First-strand cDNA was synthesized from total RNA using the reverse transcription system (Promega), then PCR for choline kinase-1 (CK1) and -2 (CK2), CTP: phosphocholine cytidylyltransferase α (CCTα), DAG: CDP-choline phosphotransferase (CPT) and GAPDH was performed. The primers used in these assays were: CK1, forward: 5′CTGAAGTACCCCACCAGGAA3′; reverse: 5′ATGCAAGGGCAAACCTATTG3′, CK2, forward: 5′TCATGCTGGTTGACTTCGAG 3,′ reverse: 5′GCCAGGTAATGGCGAATAAA3′; CCT α, forward: 5′TGCTCCAACACAGAGG ACAG 3′, reverse: 5′ACTTTGTCAACCCGTTCCTG3′; CPT1, forward: 5′GCGCTTAGGAA CTCATCCTG3′, reverse: 5′AACCTCAACACGCCTGAAAC3′; GADPH, forward: 5′TCC ACCACCCTGTTGCTGTA3′, reverse: 5′ACCACAGTCCATGCCATCAC3′.

Transfections

To determine whether TonEBP was involved in membrane phospholipid synthesis, TonEBP gene was silenced previous to labeling experiments. Gene silencing was performed by using the siRNA duplex (Invitrogen) designed by Na et al. (37) (5′AUGGGCGGUGCUUGCAGCUCCUU3′/5′GGAGCUGCAAGCACCGCCCAUUU3). MDCK cells were transfected with siRNA duplex by means of Lipofectamine™ 2000 (Invitrogen) according to the manufacturer's protocol, and 18 h after transfection, cells were subjected to hypertonic NaCl medium for an extra 12, 24, and 48 h. Then cells were collected as described above and used for Western blot and phospholipid biosynthesis assays.

Microscopy

Cells were cultured as described above but on glass coverslips. After treatment, cells were washed twice with sterile PBS, fixed with 3.7% paraformaldheyde in PBS for 30 min, and permeabilized with 0.1% Triton-X100 in PBS for 30 min. Fixed cells were incubated with a mixture of 1 μg/ml phalloidin-FITC conjugate (Sigma-Aldrich), 20 μg/ml Concanavalin A, Alexa Fluor® 594 conjugate (Molecular Probes®), and 2.5 μM Hoechst 33258 (Sigma-Aldrich) for 30 min. After labeling, samples were exhaustively washed with PBS and mounted with a drop of Vectashield mounting medium (Vector Laboratories). Differential Interference Contrast (DIC) and fluorescence images were obtained with a new but different microscopy, a Nikon Eclipse Ti (with an objective Plan apo VC 60×, 1.4 DIC 1/2) with acquisition software Micrometrics SE Premium (Accu-Scope).

Statistical analysis

The results were expressed as mean ± SEM. Data from controls and different treatments were analyzed by ANOVA, and significant differences were assessed by a posteriori Scheffé test ($P < 0.05$).

RESULTS

Hyperosmolality induces phospholipid accumulation in MDCK cells

Phospholipids are unique molecules in determining the structural matrix of all cell membranes by forming the phospholipid bilayer (23). Thus, we determined the mass of phospholipid per cell to estimate the extent of cellular membrane. We first evaluated whether an increase in osmolality affects phospholipid content. Because the hyperosmolality of the renal medullary insterstitium is made by variable and high concentrations of NaCl and urea (6, 13), we used both to prepare hyperosmotic media. The experiments were performed in the MDCK cell line, which has been typified as a transporting-polarized epithelium that possesses both morphological and enzymatic properties of cells from distal tubules and medullary collecting ducts of the kidney (28, 29). Because MDCK cells submjected to high-sodium hyperosmotic medium behave as inner medullary-collecting duct cells, they are usually used as a model system for studying medullary cell physiology and adaptation to osmotic stress (32, 38–40). MDCK cells were cultured with commercial medium alone (isosmolar condition, 298 mosmol/kg H_2O) or supplemented with a mix of different concentrations of sterile NaCl and urea in order to reach osmolalities between 300 and 760 mosmol/kg H_2O (see table inserted at the bottom of Fig. 1). As shown in Fig. 1, the increase of the osmolality of the culture media significantly increased phospholipid mass (Fig. 1A). After 24 h of treatment, incubation media with osmolalities of 585, 620, and 760 mosmol/kg H_2O significantly augmented phospholipid content by 36, 69, and 97%, respectively. Such an effect was even higher after 48 h of treatment; phospholipid mass significantly increased by 73, 97, and 135%, respectively (Fig. 1A). The increase in phospholipid mass was dependent not only on the medium osmolality but also on the time that cultures were incubated with a particular osmolality. As seen in Fig. 1B, for each osmolality assayed, the content of phospholipid per 10^6 cells after 48 h of incubation was significantly higher than that observed at 24 h. Because phospholipids almost exclusively build cellular membranes, we can speculate that changes in osmolality induce the augment in membrane extension.

To determine which solute, NaCl, urea, or both, was responsible for these effects, we cultured MDCK cells with medium containing different concentrations of NaCl, urea, or a mix of both (see Table in Fig. 2) for 24 h. Similar to that observed in Fig. 1, the content of total phospholipid per cell was augmented as a function of the osmolality, but such a response was only observed in cells cultured with high NaCl media. Thus, the addition of 100 mM NaCl to the commercial medium (A: ~475 mosmol/kg H_2O) induced a 41% increase in total phospholipid mass; of 125 mM NaCl (B: ~512 mosmol/kg H_2O) increased phospholipids by 80%; and the highest NaCl concentration assayed (C: 150 mM, ~580 mosmol/kg H_2O) caused a 117% increase. Similar results were found when cells were incubated in the presence of different concentrations of NaCl and urea. However, no differences in phospholipid mass were found after treatment with urea alone (Fig. 2A). The mass of protein per cell was also augmented as a function of sodium concentration; but, unlike that observed for phospholipids, 125 mM NaCl (~512 mosmol/kg H_2O) was required to induce a significant change (Fig. 2B). Figure 2C shows how variations in media osmolality affected the number of cells collected after treatment. As can be seen in the inset in Fig. 2C, the cells that survived to the treatment showed viabilities higher, to 90%. The finding that cell number decreases as a function of the osmolality is consistent with previous reports showing that the elevation of NaCl to over 400–500 mosmol/kg H_2O decreases the cell number mainly due to the decrease in the proliferation rate as a consequence of cell cycle arrest and, to a lesser extent, to the apoptotic process (34, 41). To discard the possibility that most of the cells were arrested in the S or G2/M phases, which could explain per se the increase in phospholipid and protein mass, we determined the effect of 125 mM NaCl (~512 mosmol/kg H_2O) on cell cycle distribution after 24 h and 48 h of treatment. **Figure 3** shows a representative histogram (Fig. 3A) and its quantification (Fig. 3B). As seen, most of the cells were found in the G1 phase, independent of the treatment applied. After 24 h and 48 h of treatment, the number of cells in the G1 phase was 77% and 85% in isotonicity and 69% and 75% in hypertonicity, respectively. Cells in the S phase decreased from 11% after 24 h of isotonicity to around a 5% in hypertonicity after 24 h and 48 h, whereas G2/M arrest was only slightly increased by NaCl treatment.

In a previous work, it was demonstrated that hypertonicity induces MDCK differentiation that includes changes in cell shape, increase in cell size, and the complete generation of apical and basolateral membrane domains (32). These previous observations are consistent with the osmotic-induced increase in phospholipid mass shown in Fig. 2A. The augmentation in cell size may reflect the increase in the cellular membrane extension. To visualize this, MDCK cells were grown on coverslips as described in Materials and Methods, subjected to 125 mM NaCl for 24 h and 48 h, and stained with phalloidin-FITC conjugate, Concanavalin A-Alexa Fluor® 594 conjugate, and Hoechst 33258. Phalloidin binds to F-actin placed close to the plasma membrane; thus, phalloidin staining allows inferring cell shape and size, Concanavalin A stains endoplasmic reticulum and Golgi membranes, thus allowing inferring endomembrane content. As seen in Fig. 3C, 24 h and 48 h of hypertonicity induced changes in cell shape and clearly increased endomembrane labeling.

The accumulation of phospholipids is an early response to hyperosmotic stress

We next evaluated the kinetics of the NaCl-induced phospholipid mass increase. It was reported that the incubation of renal epithelial cells with high NaCl-containing media (osmolalities over 600 mosmol/kg H_2O) induces oxidative stress causing DNA breaks and protein oxidation, among other injuries (42). Thus, to avoid these possible deleterious effects, which could interfere with the objectives of this work, the experiments herein were performed by incubating MDCK cultures with hyperosmotic media that did not exceed 125 mM (512 mosmol/kg H_2O). The experiments shown in **Fig. 4** were performed by incubating MDCK cells with commercial medium supplemented with 125 mM NaCl for different periods of time. This sodium concentration allowed us to find a significant increase in phospholipid content and also to collect an adequate number of cells after 24 h of treatment (Fig. 2). As seen in Fig. 4, the NaCl-induced phospholipid increase was dependent on the time of the treatment. Hyperosmolality induces a steady accumulation of phospholipid mass reaching a maximum level between 24 h and 48 h of treatment. The increase in phospholipid content was 49, 72, 120, and 104% after 12, 24, 48, and 72 h, respectively (Fig. 4A); longer incubation times did not cause a higher increase in phospholipid or in protein mass (not shown). Hyperosmolality increased protein mass by 85, 134, and 90% after 24, 48, and 72 h (Fig. 4B). Although the incubation with high-sodium medium caused time-dependent growth of phospholipid and protein masses, a phospholipid increase occurred earlier. This finding is of functional relevance, because within the first hour of exposure to high-NaCl medium, cells activate osmoprotective gene expression, such as that of osmolyte transporters and other membrane-bound proteins involved in osmoprotection (13, 43). This is the case with COX-2, which has been reported as an osmoprotective protein (10, 44). In addition, COX-2 is a regulator of renal medullary phospholipid synthesis and membrane homeostasis (45, 46). As seen in Fig. 4C, the significant osmotic-induced upregulation of COX-2 protein, which occurred after 12 h of incubation with high-NaCl medium, was accompanied by an increase in phospholipid mass. These results lead us to speculate that the early accumulation of phospholipids, which are the main constituents of cellular membranes, may be a requisite for the adequate implementation of defensive strategies, because they could be necessary for the insertion of membrane-bound osmoprotective-induced proteins. The hyperosmolar-induced accumulation of phospholipids and proteins is consistent with the observed increase of cell size (Fig. 3).

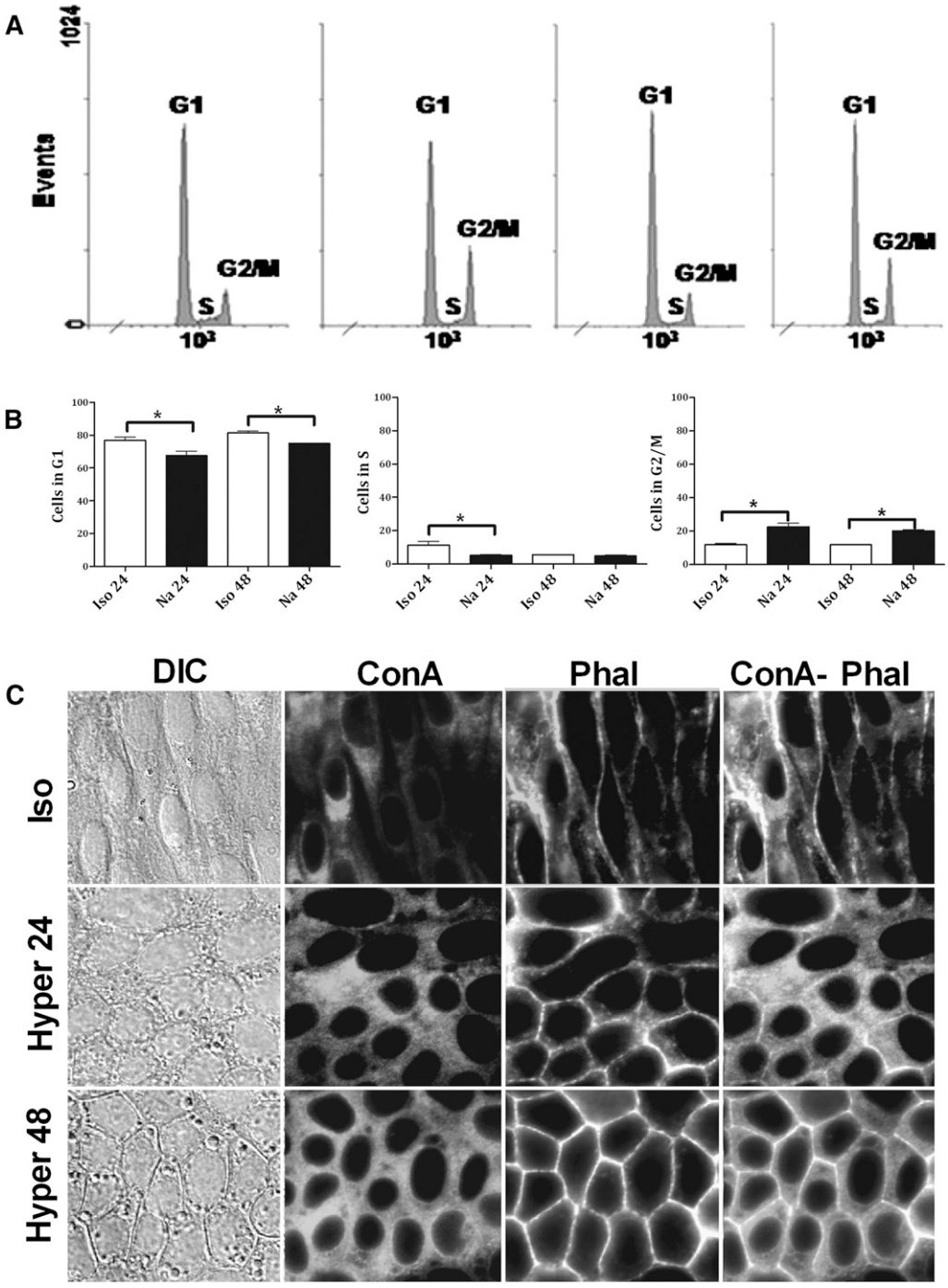


Fig. 3. Effect of hyperosmolality on cell cycle distribution, cell shape, and membrane extension. MDCK cells were grown in a mixture containing DMEM/Ham's-F12 (1:1), 10% FBS, and 1% antibiotic mixture. After reaching 70–80% confluence, cells were placed in low-serum medium (0.5% FBS) for 24 h and then subjected to 125 mM NaCl for 24 h and 48 h. After treatment, cells were collected and fixed with sterile ice-cold ethanol (70%) and stained with propidium iodide as described in Materials and Methods for flow cytometry analysis. A: Representative histogram of cell cycle distribution; B: Quantification of the histograms of three independent experiments. *Significantly different from isosmolar control values (Iso, 298 mosm/kg H_2O) at 24 h and 48 h, $P < 0.05$. C: In another set of experiments, MDCK cells were grown on sterile coverslips in a mixture containing DMEM/Ham's-F12 (1:1), 10% FBS, and 1% antibiotic mixture. After reaching 70–80% confluence, cells were placed in low-serum medium (0.5% FBS) for 24 h and then subjected to 125 mM NaCl for 24 h and 48 h. After treatment, cells were fixed and stained with a mixture of Phalloidin-FITC, Concanavalin A-Alexa Fluor® 594, and Hoechst as described in Materials and Methods. After labeling, samples were exhaustively washed with PBS and mounted with a drop of Vectashield mounting medium. DIC and fluorescence images were obtained with a new but different microscopy, a Nikon Eclipse Ti with acquisition software Micrometrics SE Premium (Accu-Scope). The figure shows a representative image of two independent experiments.

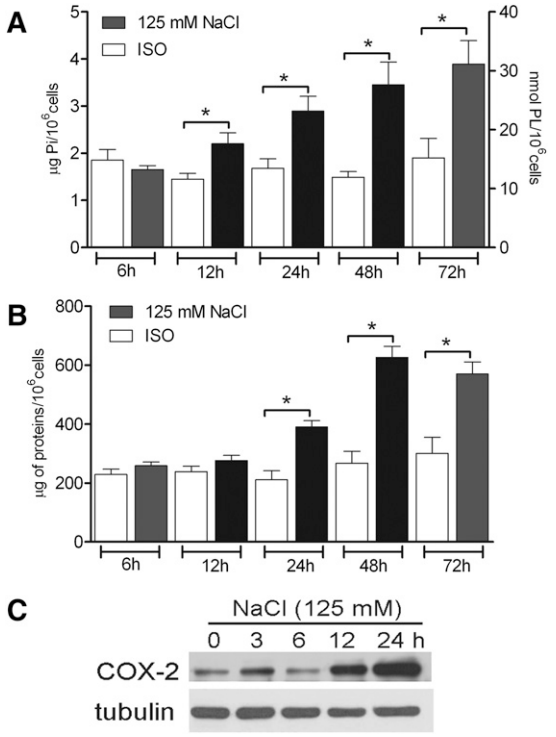


Fig. 4. Hyperosmolar-induced phospholipid increase is an early event in cell adaptation to osmotic changes. MDCK cells were grown in a mixture containing DMEM/Ham's-F12 (1:1), 10% FBS, and 1% antibiotic mixture. After reaching 70–80% confluence, cells were placed in low-serum medium (0.5% FBS) for 24 h and then subjected to high-NaCl medium (125 mM, ~512 mosm/kg H_2O) for different periods of time. After treatment, cells were collected and counted, and then used for phospholipid and protein determinations as described in Materials and Methods (A and B, respectively). Results express the mean ± SEM of five independent experiments. *Significantly different from isotonic value $P < 0.05$. C: Expression of the cytoprotective protein COX-2 as a function of the time of hyperosmolality. The Western blot image is representative of three independent experiments.

Hyperosmolality modulates membrane phospholipid composition

To evaluate whether high-NaCl medium induced quantitative or qualitative changes of phospholipid mass, we next determined the content of each phospholipid and its relative contribution to the membrane composition in MDCK cells incubated for 6, 12, 24, 48, and 72 h with 125 mM NaCl. Cells were collected, and phospholipidic species were separated by TLC and quantified as described in Materials and Methods. The change in phospholipid mass (expressed as ng of Pi/10^6 cells **Table 1**) at each experimental time was expressed as the ratio between the mass of each phospholipid in hypertonic cells and isotonic cells per 100 (%). The asterisks on the bars represent significant differences between each time of treatment and zero time (non-treated cells) (**Fig. 5A**). As seen, hyperosmolality caused asymmetric variations in the quantity of individual phospholipids. The mass of phosphatidylcholine (PC) and phosphatidylethanolamine (PE), the major glycerophospholipids of the bilayer, increased 58, 95, 133, and 118% and 22, 70, 98, and 140% after 12, 24, 48, and 72 h of hyperosmolality, respectively (Fig. 5A, PC and PE panels; Table 1). Sphingomyelin (SM), a sphingophospholipid that importantly contributes to the lipid bilayer structure, increased its content 95, 125, 132, and 110% after 12, 24, 48, and 72 h, respectively (Fig. 5A, SM panel and Table 1). The minor phospholipid phosphatidylserine (PS) was the most-sensitive membrane constituent to hyperosmolality; its mass was augmented by 78% after 6 h of treatment (Table 1). The maximal mass accretion for PS was observed after 12 h of hypertonic treatment (145%), and then it diminished with incubation time (Fig. 5A, PS panel). The other minor phospholipid, phosphatidylinositol (PI), increased 58, 130, and 65% after 24, 48, and 72 h of high-NaCl media (Fig. 5A, PI panel). Therefore, the increase found after 12 h of hyperosmolality in total phospholipid mass occurred as a consequence of the accretion of PC, the key lipid contributor to the bilayer formation, and SM, which, in polarized epithelial cells, is mainly found in the apical membrane (20). The sharp increase found in PS content at early stages of treatment may be related to an apoptotic process triggered by the exposure to high-NaCl medium (47).

The contribution of each phospholipid to total membrane phospholipid content was evaluated by calculating the ratio between the content of each phospholipid in relation to total phospholipid mass per 100 (%). When both isosmolar and hyperosmolar treatments were compared, we found that the individual phospholipid contribution to the lipid bilayer changed as a function of the incubation time. After 72 h of treatment, isosmolality and hyperosmolality, the choline-containing phospholipids PC and SM concentration in the phospholipid bilayer increased whereas PE and PI concentration decreased (Fig. 5B). However, the kinetics of such variations were different. Both treatments significantly increased the relative concentration of PC in the bilayer from 45.4% to 53.9% between 6 h and 72 h of incubation, respectively. However, the maximal PC concentration in isosmolality was found after 72 h, whereas in hyperosmolality, this occurred just after 24 h of treatment (Fig. 5B, PC panel). Similar behavior was observed for SM, which varied from 11.8% to 19% between 6 h and 48 h in hyperosmolality as well as in isosmolality (Fig. 5B, SM panel). But, in hyperosmolality, such an increase was found after 12 h, whereas in isosmolality, cells required 48 h to reach a similar SM concentration (Fig. 5B, SM panel). In both cases, after 48 h, the SM concentration fell to 16%. In contrast, both treatments decreased the relative concentrations of PE, PI, and PS with incubation time. Thus, PE concentration fell from 25.3% to 20.1% between 6 h and 72 h, respectively. PI diminished from 10% to 6% after 6 h and 72 h, respectively (Fig. 5B, PI panel). Under isotonic conditions, the relative concentration of PS was maintained around 5% at all incubation times (Fig. 5B); hyperosmolality increased PS relative concentration to 9% and 8.5% after 6 h and 12 h of treatment; thereafter, the percentage of PS decreased, returning to isosmotic values (Fig. 5B).

In our experiments, cells were incubated until 72 h after confluence. Under these conditions, it has been reported

TABLE 1. Phospholipid content in hypertonic-treated MDCK cells

	Phospholipid Content											
	ng of Pi/10^6 cells											
	6 h		12 h		24 h		48 h		72 h		96 h	
	I	H	I	H	I	H	I	H	I	H	I	H
PC	367.6 ± 24.2	302.3 ± 41.2	401.8 ± 31.3	577.9* ± 54.2	394.0 ± 34.8	767.2* ± 61.1	394.0 ± 48.3	916.1* ± 71.2	450.5 ± 21.2	977.7* ± 79.2	505.8 ± 24.2	968.1* ± 80.1
PE	194.2 ± 21.3	170.7 ±19.9	198.1 ±18.9	243.0 ± 34.1	165.6 ±15.2	282.1* ±19.2	133.0 ±16.1	264.4* ±31.2	158.9 ±19.7	382.3* ±58.3	206.6 ±31.3	421.3* ±64.2
SM	89.0 ± 11.0	80.1 ± 9.3	101.2 ±10.8	177.9* ± 27.4	122.2 ±16.4	261.9* ± 29.3	139.3 ±15.0	291.5* ±30.1	142.3 ±17.3	303.4* ±34.1	118.3 ±13.3	214.5* ±28.3
PI	77.2 ± 9.3	65.6 ± 5.2	69.0 ±11.3	74.3 ± 12.1	57.2 ±11.1	82.5 ±15.3	45.6 ± 9.3	108.9* ±9.3	69.2 ±11.3	112.3* ±11.0	49.0 ±9.3	105.7* ±15.1
PS	39.2 ± 6.5	61.3* ± 9.3	38.0 ±5.3	99.9* ± 11.3	41.2 ± 7.8	77.3* ± 21.3	34.5 ± 6.3	61.2* ±8.8	45.2 ±7.0	70.0* ±6.3	45.3 ±4.6	70.2* ±9.7

MDCK cells were incubated in commercial medium (I, isotonicity, 298 ± mosmol/kg H_2O) or in medium supplemented with 125 mM NaCl (H, hypertonicity, 512 ± 12 mosmol/kg H_2O) for different periods of time. At each time, cells were collected and counted, and phospholipid was extracted and separated by TLC as described in Materials and Methods. The results are expressed as ng of Pi/10^6 cells and represent the mean ± SEM of independent experiments.

*Hypertonic values significantly different from isotonic values, $P < 0.05$.

that MDCK cells acquire a polarized differentiated phenotype that involves the generation of basal and apical membrane domains (20, 48). Recently, it has been demonstrated that hyperosmolality induced MDCK differentiation after 48 h of incubation; under these conditions, high NaCl regulates the formation of glycosphingolipids and the formation of the apical membrane domain (32). Thus, the phospholipid profile found after 72 h of incubation could correspond to a differentiated stage. Our data clearly showed that the final composition of polarized MDCK cell membrane phospholipids was anticipated by hyperosmolar treatment. The present results agree with previous work showing that hyperosmolality induces the early accumulation of SM, a main constituent of apical-membrane (20), and increases the concentration of PC, the key lipid in lipid bilayer formation, needed for the expansion of the basolateral domain and a key factor in renal cell differentiation (4, 32).

Hyperosmolality causes a biphasic regulation of phospholipid de novo synthesis

We next evaluated phospholipid biosynthesis in MDCK cells subjected to hyperosmolality. Phospholipid de novo synthesis begins when a molecule of *sn*-glycerol 3P is successively acylated to be transformed into phosphatidic acid (PA); PA is then converted in diacylglycerol (DAG), which enters to the CDP-choline/ethanolamine pathway to form PC and PE, or in CDP-DAG, which is the precursor for PI synthesis (see scheme of the biosynthetic pathway in **Fig. 6**) (22, 49). Therefore, the incorporation of [U^{14}C]glycerol (^{14}C-Gly) into phospholipids allows monitoring the de novo synthesis, because it is incorporated at the beginning of the biosynthetic process. In isosmotic conditions, the highest ^{14}C-Gly incorporation was found associated to PC (5,600 ± 700 dpm/10^6 cells) and PA (1,950 ± 126 dpm/10^6 cells), followed by PI (1,030 ± 112 dpm/10^6 cells) and PE (553 ± 96 dpm/10^6 cells), whereas no label was found associated to PS under our experimental conditions. Under hyperosmolality, the incorporation of ^{14}C-Gly into membrane phospholipids exhibited a biphasic kinetic (Fig. 6). During the first 12 h, the ^{14}C-Gly labeling of phospholipids dropped to around 50% of isosmolar values. The radioactivity associated to PC represented 66, 63, 63, and 48% of isosmolar values after 3, 6, 9, and 12 h of hypertonic treatment, respectively. Similar results were found when PE and PI labeling were analyzed. In contrast, the incorporation of ^{14}C-Gly into PA under hypertonic conditions remained similar to isotonic values. After 24 h, phospholipid de novo synthesis not only recovered control levels but also was sharply augmented. The incorporation of ^{14}C-Gly increased by 88, 195, and 165% into PC, by 54, 270, and 172% into PE, and by 225, 259, and 190% into PI after 24, 48, and 72 h of hyperosmolar treatment, respectively (Fig. 6A). The increase in the incorporation of ^{14}C-Gly into PA was of 24, 182, and 105% after 24, 48, and 72 h, respectively. These results explain the mass increase observed after 24 h of treatment with high NaCl. However, they do not make clear why the phospholipid mass is augmented after 12 h. Considering that after 12 h of treatment, the

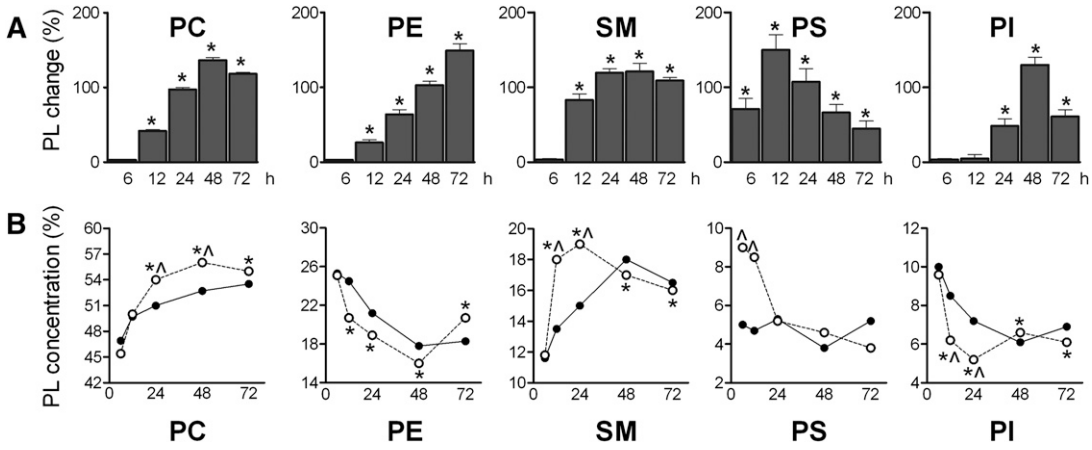


Fig. 5. Effect of hyperosmolality on membrane composition and phospholipid profile. MDCK cells were grown in a mixture containing DMEM/Ham's-F12 (1:1), 10% FBS, and 1% antibiotic mixture. After reaching 70–80% confluence, cells were placed in low-serum medium (0.5% FBS) for 24 h and then subjected to high-NaCl medium (125 mM, ~512 ± 12 mosm/kg H_2O) for different periods of time. After treatment, cells were collected, counted, and then used for phospholipid determination. Phospholipids were extracted and separated by TLC as described in Materials and Methods. Each phospholipid was determined comparing with the specific standard, scraped off of the TLC, and quantified as described in Materials and Methods. A: The graphs represent the change in the mass of each phospholipid in hyperosmolality with respect to isosmolality expressed as percentage (%) at the time of each treatment. Results express the mean ± SEM of five independent experiments. *Significantly different from isotonic values, $P < 0.05$. B: The graphs represent the concentration of each phospholipid in the total phospholipid content expressed as percentage (%) at the time of each treatment. Closed circles correspond to isosmolality and open circles to hyperosmolality. Results express the mean ± SEM of five independent experiments. *Significantly different from isotonic value at 6 h of incubation. ^Significantly different from the corresponding isotonic value, $P < 0.05$. PC, phosphatidylcholine; PE, phosphatidylethanolamine; SM, sphingomyelin; PS, phosphatidylserine; PI, phosphatidylinositol.

mass increase is due to PC and SM (Table 1), which are involved in signaling processes, one explanation could be that degradatory activities were reduced by hypertonic treatment and, as a consequence, an increase in both phospholipids occurs. Another possibility is that the early PC increase does not occur by the de novo synthesis of PA but utilizes DAG coming from cellular storage. However, it is yet to be proven.

When the distribution of the label per phospholipid was analyzed, we found that most of the ^{14}C-Gly radioactivity was associated to PC (61.3% of total label) and PA (21.3% of total label), followed by PI (10.4%) and PE (6.4%) (**Table 2**). During the first 12 h of treatment, the ^{14}C-Gly radioactivity delivered to PC and PE decreased by around 20%; such radioactive precursor was kept in the biosynthetic precursor PA, which increased in the same magnitude. Thus, hyperosmolality may repress the conversion of PA into DAG in the de novo biosynthetic pathway or may deliver the de novo-synthesized DAG to triacylglycerol synthesis. After 24 h of treatment, phospholipid synthesis was reactivated and the flow of ^{14}C-Gly radioactivity to PC and PE was restored. Similarly, the molecules of ^{14}C-Gly radioactivity delivered to PI increased around 100% with the concomitant diminution of PA (Table 2). The fact that part of the labeling of PA was relocated to PI and PC indicates the priority of the cells to replenish the membranes with these phospholipids. These results clearly show a differential regulation of the biosynthetic process by hyperosmolality. Whether such an effect occurs is under study in our laboratory.

To confirm that the hyperosmolality-induced membrane biogenesis is due to the increase in NaCl and not to urea, we evaluated the incorporation of ^{14}C-Gly radioactivity to phospholipids after 24 h of treatment with NaCl, urea, and the mix of both. As seen in Fig. 6B, the increase in phospholipid synthesis was stimulated by NaCl and the mix of NaCl and urea but not by urea alone. This result confirms that hyperosmotic-induced membrane biogenesis is exclusively due to sodium action.

Hyperosmolar-induced phospholipid biosynthesis is mediated by MAPKs

It is well documented that mitogen-activated protein kinase (MAPK) pathway activation mediates adaptive mechanisms against environmental tonicity in renal cells (11, 13, 50, 51). In addition, we demonstrated that the maintenance of phosphatidylcholine synthesis is mediated by ERK1/2 MAP kinase in adult renal medullary cells (52). Thus, we next evaluated whether such a pathway could be involved in the hyperosmotic regulation of the synthesis of this major phospholipid, PC. For this purpose, cells were pretreated with the specific MAP kinase kinase inhibitor U0126 for 30 min before NaCl addition. To evaluate the initial and late stages of PC synthesis and turnover, we studied the incorporation of ^{32}P-orthophosphate (^{32}P-Pi) into PC biosynthesis after 3 h and 24 h of hyperosmolality (**Fig. 7**). The use of ^{32}P-Pi labeling allowed us to monitor phospholipid de novo synthesis, that occurred from the *sn*-glycerol 3P, and phospholipid resynthesis, that occurred from the DAG generated by phospholipases action on membrane phospholipids (18, 53). As seen in Fig. 7A, U0126 blocked the effect of NaCl on PC synthesis after 3 h and 24 h of treatment. We also measured the effect of hypertonicity on the activation of ERK 1/2 MAP kinase by

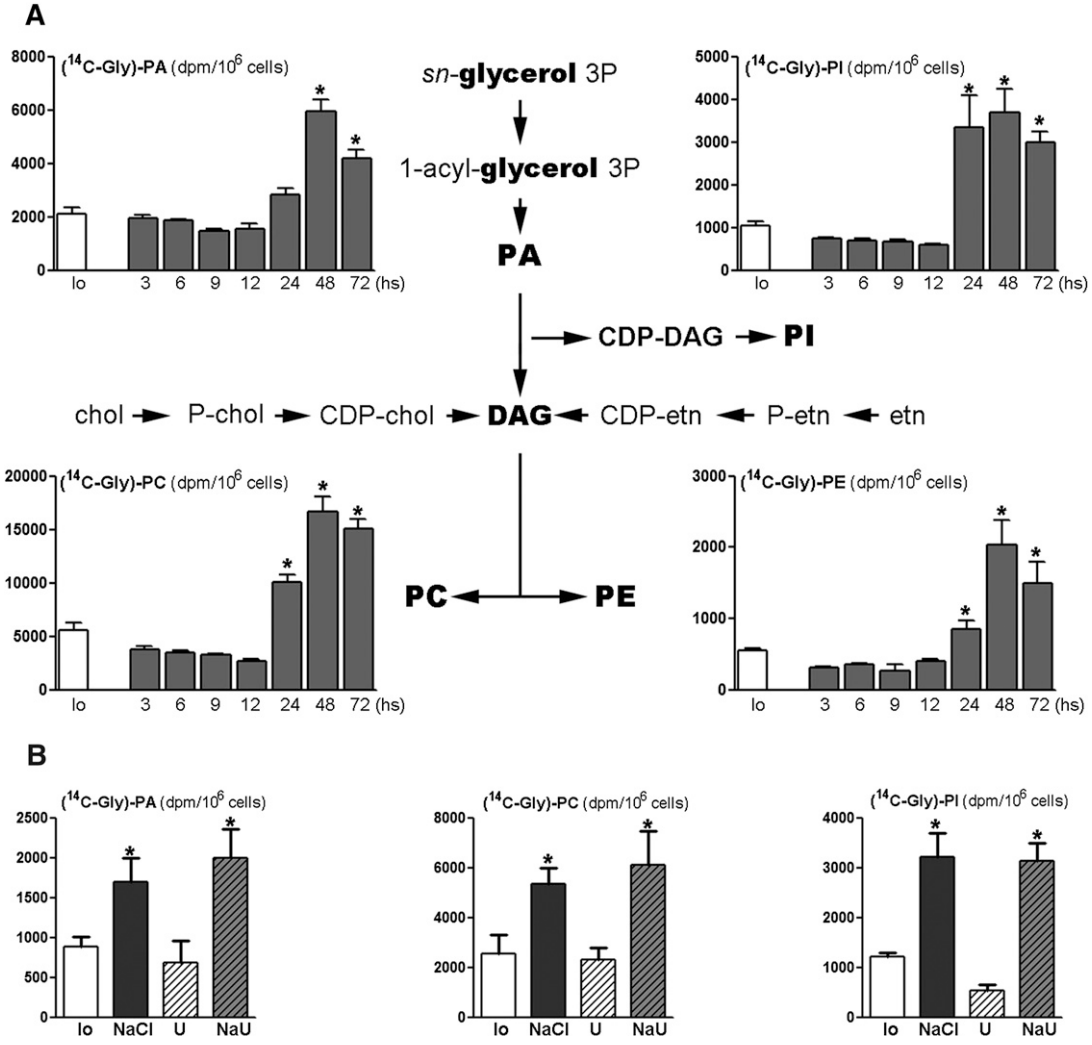


Fig. 6. Hyperosmolality activates phospholipid de novo synthesis. MDCK cells were grown in a mixture containing DMEM/Ham's-F12 (1:1), 10% FBS, and 1% antibiotic mixture. After reaching 70–80% confluence, cells were placed in low-serum medium (0.5% FBS) for 24 h and then subjected to high-NaCl medium (125 mM, ~512 ± 12 mosm/ kg H_2O) for different periods of time. To evaluate the generation of new phospholipid molecules, cells were incubated in the presence of 2 μCi/ml [U-^{14}C]glycerol for 3 h. After treatment, cells were collected and counted, lipids were extracted, phospholipid species were separated by TLC, and the radioactivity incorporated in each phospholipid was visualized by radioautography and quantified by liquid scintillation counting. A: White bars: isotonicity; gray bars: hypertonicity. B: Effect of NaCl, urea, or both on phospholipid de novo synthesis. Data represent the mean ± SEM of four independent experiments. *Significantly different from control values (Iso, 298 mosm/kg H_2O), $P < 0.05$.

determining enzyme phosphorylation by Western blot analysis (Fig. 7B). As seen, 3 h of treatment increased the levels of P-ERK1/2, and such an increase was blocked by U0126. This finding was coincident with that observed for PC synthesis after 3 h of stimulation with NaCl. In contrast, 24 h of NaCl treatment did not increase the levels of P-ERK1/2, but the increase in PC synthesis after 24 h of treatment was still blocked by U0126. This finding led us

TABLE 2. ^{14}C-glycerol distribution in individual phospholipids

		Hours of Treatment							
		0	3	6	9	12	24	48	72
Distribution of ^{14}C-Glycerol (%)	PA	23.1 ± 2.4	28.7 ± 2.5	28.7 ± 2.3	26.5 ± 1.7	28.9 ± 1.8	9.1 ± 2.7	19.2 ± 1.9	17.0 ± 2.2
	PC	64.0 ± 2.1	55.8 ± 1.6	54.6 ± 1.3	57.1 ± 2.1	50.0 ± 1.9	63.4 ± 2.3	59.1 ± 1.5	61.2 ± 2.8
	PE	6.1 ± 1.2	4.4 ± 1.1	5.4 ± 1.6	4.7 ± 0.8	7.4 ± 1.2	6.3 ± 1.3	7.1 ± 1.7	6.7 ± 1.2
	PI	8.2 ± 0.9	10.8 ± 1.5	10.9 ± 1.7	11.5 ± 0.9	11.5 ±1.3	21.1 ± 4.5	13.2 ± 2.5	13.4 ± 1.8

Cells were treated with high NaCl for different periods of time, and 3 h before collecting, were incubated in the presence of 2 μCi/ml [U-^{14}C] glycerol (^{14}C-glycerol). Phospholipids were extracted and separated as described in Materials and Methods, and the radioactivity incorporated was visualized by radioautography and quantified by liquid scintillation counting. The distribution of radiolabeled glycerol into phospholipids was calculated as radioactivity incorporated into PA, PC, PE, or PI (CPM)/total radioactivity incorporated into phospholipids (CPM) × 100.

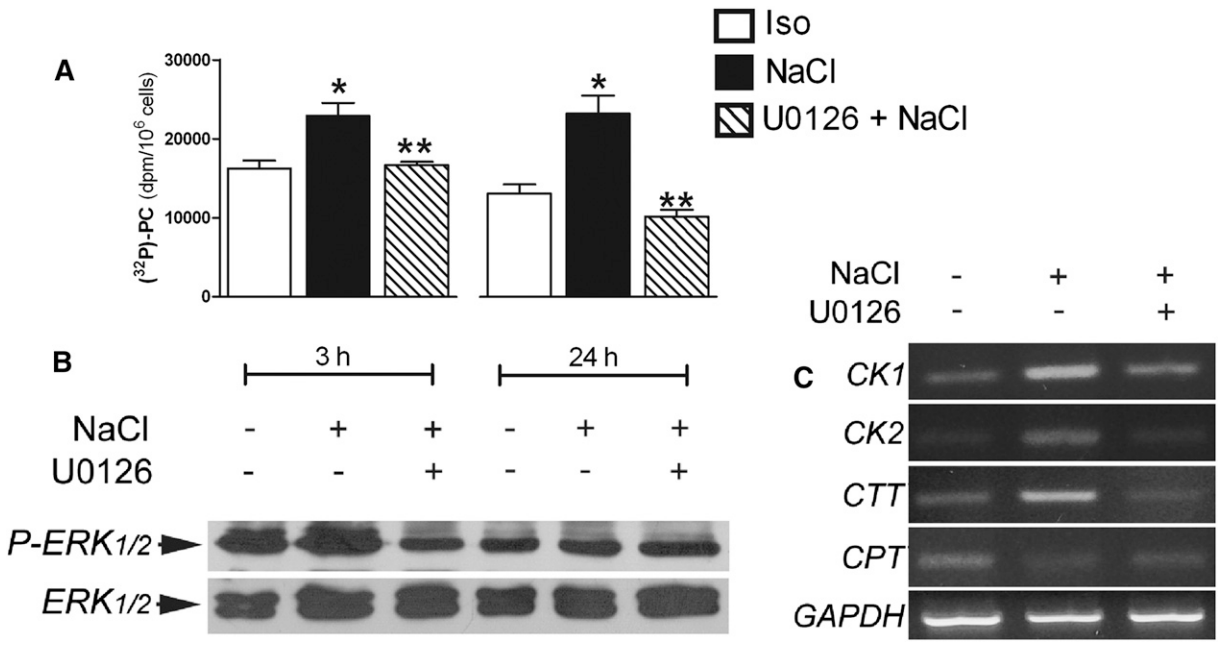


Fig. 7. Hypertonic-induced phosphatidylcholine synthesis is dependent on the ERK1/2 MAPK pathway. MDCK cells were grown in a mixture containing DMEM/Ham's-F12 (1:1), 10% FBS, and 1% antibiotic mixture. After reaching 70–80% confluence, cells were placed in low-serum medium (0.5% FBS) for 24 h and then subjected to high-NaCl medium (125 mM, ~512 ± 12 mosm/kg H_2O) for 3 h and 24 h in the absence or in the presence of 1 μM U0126. A: Three hours before cell harvesting, 1 μCi/ml of [^{32}P]orthophosphate carrier-free was added to the cultures to label phosphatidylcholine. After treatment, cells were collected and counted, lipids were extracted, PC was separated by TLC, and the radioactivity incorporated was visualized by radioautography and quantified by liquid scintillation counting. White bars, isotonicity; black bars, NaCl alone; hatched bars, U0126 + NaCl. Data represent the mean ± SEM of four independent experiments. *Significantly different from control values (Iso, 298 mosm/kg H_2O), $P < 0.05$; **significantly different from hypertonic values, $P < 0.05$. B: Western blot determination of P-ERK1/2 and total ERK1/2 from cells treated as described above. The image is representative of two independent experiments. C: RT-PCR determination of CK1 and CK2, CCTα, and CPT mRNAs from cells treated as previously described. The image is representative of two independent experiments.

to evaluate whether the early blockage of ERK 1/2 MAP kinase could be affecting the expression of PC biosynthetic enzymes (Fig. 7C). To do this, we evaluated the expression of mRNA of CK-1 and -2, CTP:CCTα, and DAG:CDP-CPT after 24 h of treatment with NaCl in the absence or in the presence of U0126. As can be observed, hypertonicity upregulated the expression of CK1, CK2, in lesser extent, and CCT but did not cause any change in CPT expression. U0126 treatment blocked the expression of both enzymes CK and CCT. These findings clearly demonstrated that hypertonic-induced increase in PC synthesis is due to the increase in the transcriptional activity of the PC-regulatory enzymes CK and CCT. Such an increase requires the early activation of ERK 1/2 MAP kinase.

PLC and PLD participation in hyperosmolar-induced phospholipid synthesis

As mentioned before, phospholipid biosynthesis can occur through the de novo pathway from the *sn*-glycerol 3P, and through the resynthesis pathway, from the DAG generated by phospholipase action on membrane phospholipids (18, 53). Thus, to determine whether the DAG could be originated by PLC or PLD action, we evaluated the synthesis of phospholipids in the presence of U73122 (a PLC-specific inhibitor), VU0359595 (a PLD1 inhibitor), and VU0285655 (a PLD2-specific inhibitor). We also used 1 buthanol, which indicates PLD activity by the formation of phosphatidylbuthanol (PLD transphosphatidylation activity).

As seen in **Fig. 8**, 3 h of hyperosmolality increased the incorporation of ^{32}P-Pi into PC and PI by 41% and 74%, respectively, whereas no change was observed in PA. Because no increase in phospholipid de novo synthesis was previously observed (Fig. 6), this result could indicate the activation of degradatory phospholipases, which provided the substrates DAG or PA for phospholipid resynthesis. In fact, the inhibition of PLC by U73122 blocked hypertonic-stimulated PC and PI resynthesis (Fig. 8A). The use of 1-buthanol, which indicates PLD activity, decreased PC synthesis to basal levels. The PLD1-specific inhibitor VU0359595 did not affect PC synthesis and significantly increased PI and PA labeling when compared with hypertonic values (Fig. 8A). No effect on phospholipid synthesis was observed with a PLD2-specific inhibitor.

After 24 h of incubation with high-NaCl medium, the incorporation of ^{32}P-Pi to PC, PI, and PA increased 78, 64, and 110%, respectively (Fig. 8B). In this case, and similarly to that observed with ^{14}C-Gly, there was a net synthesis of the precursor PA. PLC inhibition decreased PC, PI, and PA synthesis. Thus, hypertonic-induced phospholipid synthesis requires the activation of PLC enzyme. Considering that after 24 h, we found a net de novo synthesis of phospholipids (Fig. 6), the inhibition of PLC should cause an accumulation of PI, but this was not observed. Thus, it is probable

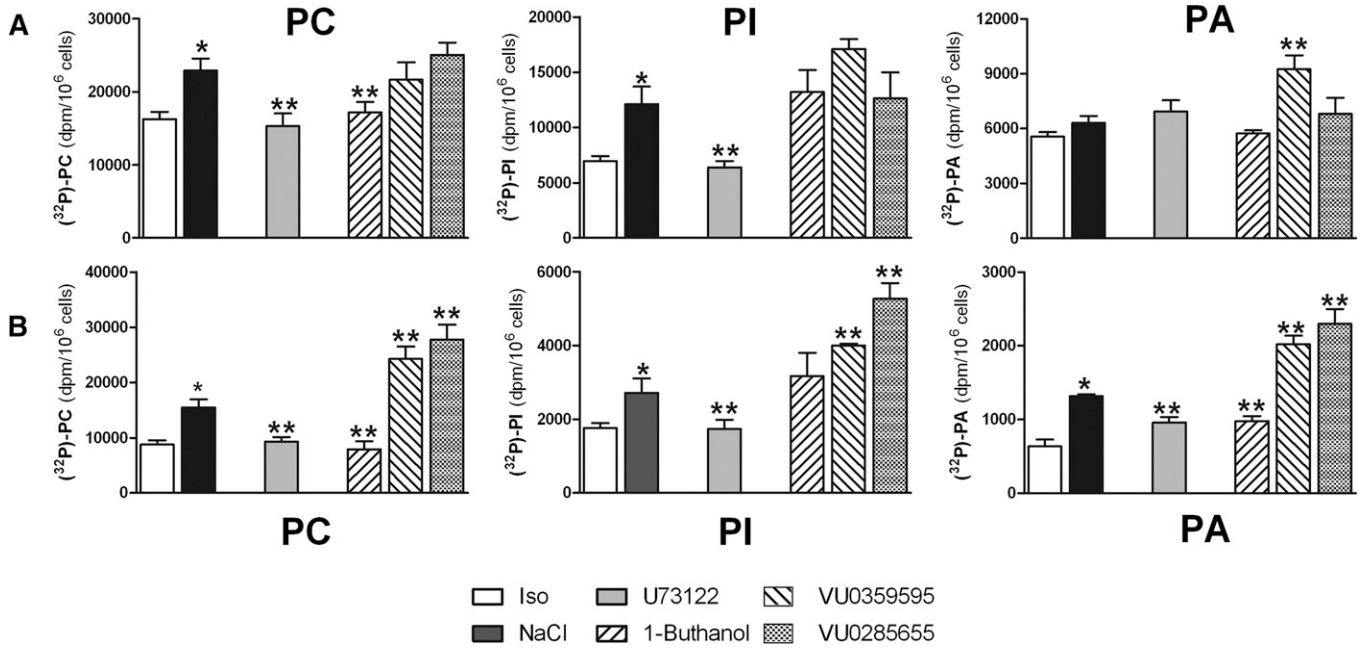


Fig. 8. PLC and PLD participation in hypertonic activation of phospholipid synthesis. MDCK cells were grown in a mixture containing DMEM/Ham's-F12 (1:1), 10% FBS, and 1% antibiotic mixture. After reaching 70–80% confluence, cells were placed in low-serum medium (0.5% FBS) for 24 h and then subjected to high-NaCl medium (125 mM, ~512 ± 12 mosm/kg H_2O) for 3 h (A) and 24 h (B). To determine which signaling pathway was involved in the activation of the phospholipid biosynthetic process, cells were pretreated for 30 min previous to NaCl addition with 1 μM U73122, 0.1% buthanol, 0.15 μM VU0359595, and 0.5 μM VU0285655-1. Three hours before cell harvesting, 1 μCi/ml of [^{32}P]orthophosphate carrier-free was added to the cultures to label phospholipid synthesis. After treatment, cells were collected and counted, lipids were extracted, phospholipid species were separated by TLC, and the radioactivity incorporated in each phospholipid was visualized by radioautography and quantified by liquid scintillation counting. Data represent the mean ± SEM of four independent experiments. *Significantly different from control values (Iso, 298 mosm/kg H_2O), $P < 0.05$; **significantly different from hypertonic values, $P < 0.05$.

that a PLC acts as part of regulatory signaling mechanism more than as DAG supplier. The treatment with 1-buthanol blocked PC and PA synthesis, and both PLD1 and PLD2 inhibitors caused a significant increase of hypertonic-stimulated phospholipid synthesis. Taken together, these results indicate the participation of PLD in the generation of precursors for phospholipid synthesis. On one hand, the primary alcohol sequestered the PA generated by PLD action, thus diminishing the pool of precursor for the synthesis. On the other hand, the blockage of PLDs by the specific inhibitors together to an increased de novo synthesis caused the accumulation of all phospholipids. However, we cannot discard the possible regulatory action of PLD as a part of a signaling pathway.

Hyperosmotic-induced phospholipid synthesis is not mediated by TonEBP

TonEBP also known as NFAT5, is a transcription factor considered a master regulator of adaptive and protective responses against hyperosmolality (2, 15). Thus, we evaluated whether this transcription factor was necessary for membrane phospholipid biosynthesis. To block the activity of TonEBP, cells were transfected with 400 nM TonEBP siRNA 24 h previous to the addition of the NaCl to the culture media as previously reported (10), and then cells were incubated for 24 h and 48 h to measure phospholipid biosynthesis. As seen in **Fig. 9A**, the knockdown of TonEBP did not affect phospholipid biosynthesis kinetics. Figure 9B shows that silencing of TonEBP effectively blocked the expression of the protein. Thus, hyperosmotic-induced membrane phospholipid biosynthesis is not regulated by TonEBP.

DISCUSSION

Apart from their leading role as structural components of all biomembranes, phospholipids regulate the function of numerous membrane-bound proteins (54, 55), contribute to membrane biophysical properties (56), and play a principal role in signal transduction pathways (57). Previous work from our laboratory showed that the renal papilla, which is the zone of the kidney with the highest osmolality, possesses the highest level of phospholipid biosynthesis when compared with the renal medulla and cortex (24). We had hypothesized that such a dynamic phospholipid metabolism in papillary cells was a consequence of the high osmolality present in the interstitial tissue, but we did not have any evidence. Therefore, in the present work, we have evaluated whether phospholipid biosynthesis in renal epithelial cells is regulated by changes in environmental tonicity. Data presented herein confirm our previous hypothesis, inasmuch as they show for the first time that phospholipid de novo synthesis, which is a parameter of membrane biogenesis, is upregulated by the increase of the osmolality of the

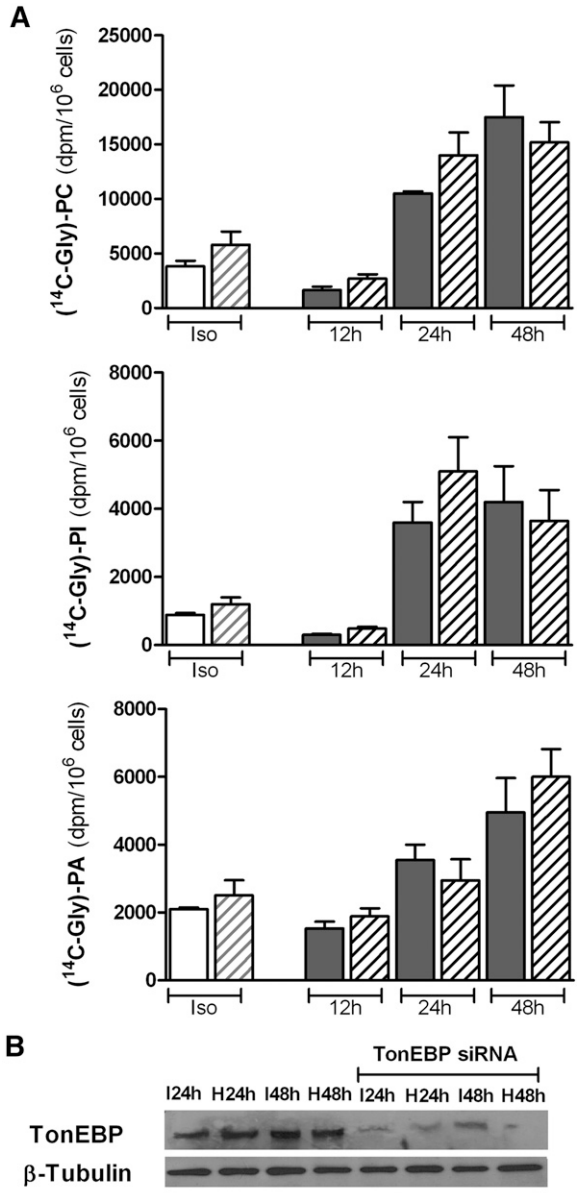


Fig. 9. Effect of TonEBP silencing on phospholipid biosynthesis. To evaluate whether TonEBP was involved in the activation of the phospholipid biosynthetic process, cells were transfected with TonEBP siRNA prior to NaCl addition. Then cells were treated for 12, 24, and 48 h with NaCl as described in Materials and Methods. After treatment, cells were collected and lipids were extracted, phospholipid species were separated by TLC, and the radioactivity incorporated in each phospholipid was visualized by radioautography and quantified by liquid scintillation counting. White bars, isotonicity; gray bars, NaCl alone; hatched bars, cells treated with TonEBP-siRNA. Data represent the mean ± SEM of two independent experiments (A). The efficiency of TonEBP silencing was evaluated by determining the levels of TonEBP protein by Western blot analysis (B).

medium. We have demonstrated that changes in the environmental osmolality increased cellular phospholipid content (Fig. 1). Such an increase was exclusively due to the variations of NaCl concentration, not to changes in urea (Fig. 2). The net augment in the endogenous phospholipid content per cell was observed after 12 h of incubation with high-sodium medium and occurred earlier than the increase in the cellular content of proteins (Fig. 4A, B). These results may suggest that cells need to expand their membrane extent as a primary response against changes in environmental tonicity. It is known that cells subjected to hyperosmotic stress upregulate the expression of osmoprotective genes (2, 7, 58). The expression of various transporters, such as sodium/myoinositol cotransporter, sodium/chloride/betaine cotransporter, and sodium/chloride/taurine cotransporter, is required for accumulation of compatible organic osmolytes that counterbalance the early increase in ionic strength (6–9). All of these are membrane-bound proteins; thus, cellular membrane expansion is an absolute requirement for cell adaptation to changes in tonicity. We have previously reported that COX-2, a membrane-bound protein, is an osmoprotective protein that is rapidly induced by the increase in NaCl concentration (10). As shown in Fig. 4C, the sharp increase in the levels of sodium-induced COX-2 occurred just after 12 h of exposure to hyperosmolality, when the content of membrane phospholipids started to increase. These findings are of physiological relevance and contribute to explaining the adaptive strategies implemented by cells when the tonicity in their environment changes. Therefore, not only must cells activate the transcription of osmoprotective proteins, they also must provide an adequate quantity of membranes.

Hyperosmolality changed MDCK cell membrane composition. After 72 h of treatment, membranes were enriched in the choline-containing phospholipids PC and SM. In contrast, the anionic phospholipids PE, PS, and PI decreased. Such a final composition was also acquired by control cells, but it occurred with a different kinetic (Fig. 5). Thus, it appears that hypertonicity anticipated the final membrane composition. It has been suggested that a hypertonic environment could regulate renal cell differentiation and renal medullary structure maturation (3). In addition, we have previously shown that hypertonicity induces redistribution of A-type lamins to nucleoplasmic speckled structures that accompany MDCK cell differentiation (59). The differentiation process requires previous cell polarization that involves an accretion of PC in the basolateral membrane and SM in apical membranes (60). In nontreated MDCK cells, polarization starts around 24 h after confluence and is completed after 3 days (61). Thus, the present results provide a new piece of evidence indicating that a hypertonic environment accelerates the renal cell polarization differentiation process because the hypertonic-induced increase of SM was found after 12 h of treatment, whereas SM accretion in nontreated cells occurred only after 48 h. The early hypertonic-induced increase in SM content could constitute an indicator of epithelial cell differentiation.

The increase in the endogenous phospholipid content was accompanied by the activation of the de novo synthesis of PC, PI, and PE (Fig. 6). During the first 12 h, the incorporation of ^{14}C-Gly into PC, PI, and PE decreased, and the labeling seemed to be accumulated in the biosynthetic precursor, PA (Table 2); but a net increase was observed only after 24 h of exposure to hyperosmolality. This result evokes the possibility that hyperosmolality regulates the conversion of PA into DAG and/or CDP-DAG, which are the next substrates in the

phospholipid biosynthetic pathway (53, 62). These results agreed with those found when ^{32}P-Pi was used as a radiolabeled precursor showing the active participation of phospholipase activities in the regulation of phospholipid synthesis (Fig. 8). In addition, it has been reported that the degradation of PC is the main source of glycerophosphocholine (GPC), a protective osmolyte (63). Thus, the rapid conversion of the newly synthesized PC into GPC could explain the lack of labeling of PC within the first hour of the hypertonic stimulus. Moreover, it is probable that the bulk of the newly synthesized PA was driven to PC and not to PE and PI so as to ensure the production of GPC. Therefore, in the early stages of hyperosmolality, the activation of phospholipid resynthesis could be focused on the necessity of producing protective osmolytes more than on expanding cellular membranes. Such a hypothesis agrees with previous reports indicating that the accumulation of the protective osmolytes occurs immediately after the exposure to hypertonicity (43). In addition, part of the newly synthesized PC could be consumed as substrate in the biosynthesis of SM, which was clearly augmented after 12 h of treatment (Fig. 5). Therefore, it is likely that in the early stages of hypertonic shock, cells activate cellular phospholipid de novo synthesis with two main purposes: to ensure the adequate quantity of membrane to host the osmoprotective proteins, and polarization.

The activation of phospholipid biosynthesis by hypertonicity includes transcriptional activation. The increase of PC de novo synthesis after 24 h of hypertonic treatment was coincident with the increase in the mRNA expression of two regulatory enzymes, CK and CCTα (49, 64), which was dependent on the activation of ERK1/2 MAP kinase. How hypertonicity activates transcriptional regulation of these enzymes is now under study in our laboratory but does not involve the transcription factor NFAT5. TonEBP/NFAT5 is a transcription factor considered a master regulator of adaptive and protective responses against hyperosmolality (2, 15). Numerous different genes involved in osmoprotection are under TonEBP transcriptional regulation (5, 10, 15). Moreover, the hypertonic-induced increase of A-type lamins, which is an indicator of a differentiated state, is also under TonEBP control (59). However, the present data showed that phospholipid de novo synthesis was not under TonEBP control (Fig. 8).

Summarizing, in the present work, the effect of hyperosmolality on membrane biogenesis was evaluated. Data presented herein show for the first time that high NaCl, but not high urea, differentially modulates the preservation of biomembranes in renal epithelial cells that could have two main purposes: to ensure the adequate quantity of membrane to host the osmoprotective proteins and the cell polarization process.

REFERENCES

1. Dmitrieva, N. I., and M. B. Burg. 2005. Hypertonic stress response. *Mutat. Res.* **569:** 65–74.
2. Kwon, M. S., S. W. Lim, and H. M. Kwon. 2009. Hypertonic stress in the kidney: a necessary evil. *Physiology (Bethesda).* **24:** 186–191.
3. Kültz, D. 2004. Hypertonicity and TonEBP promote development of the renal concentrating system. *Am. J. Physiol. Renal Physiol.* **287:** F876–F877.
4. Han, K. H., S. K. Woo, W. Y. Kim, S. H. Park, J. H. Cha, J. Kim, and H. M. Kwon. 2004. Maturation of TonEBP expression in developing rat kidney. *Am. J. Physiol. Renal Physiol.* **287:** F878–F885.
5. Woo, S. K., S. D. Lee, K. Y. Na, W. K. Park, and H. M. Kwon. 2002. TonEBP/NFAT5 stimulates transcription of HSP70 in response to hypertonicity. *Mol. Cell. Biol.* **22:** 5753–5760.
6. Burg, M. B. 1995. Molecular basis of osmotic regulation. *Am. J. Physiol.* **268:** F983–F996.
7. Burg, M. B., E. D. Kwon, and D. Kultz. 1997. Regulation of gene expression by hypertonicity. *Annu. Rev. Physiol.* **59:** 437–455.
8. Garcia-Perez, A., and M. B. Burg. 1991. Role of organic osmolytes in adaptation of renal cells to high osmolality. *J. Membr. Biol.* **119:** 1–13.
9. Xiuzhu, T., L. Shore, Y. Stram, S. C. Michaeli, H. C. Brietbart, and M. Shemesh. 2003. Duplexes of 21 nucleotide RNA specific for COX II mediates RNA interference in cultured bovine aortic coronary endothelial cells (BAECs). *Prostaglandins Other Lipid Mediat.* **71:** 119–129.
10. Favale, N. O., C. I. Casali, L. G. Lepera, L. G. Pescio, and M. C. Fernandez-Tome. 2009. Hypertonic induction of COX2 expression requires TonEBP/NFAT5 in renal epithelial cells. *Biochem. Biophys. Res. Commun.* **381:** 301–305.
11. Yang, T., Y. Huang, L. E. Heasley, T. Berl, J. B. Schnermann, and J. P. Briggs. 2000. MAPK mediation of hypertonicity-stimulated cyclooxygenase-2 expression in renal medullary collecting duct cells. *J. Biol. Chem.* **275:** 23281–23286.
12. Yang, T., I. Singh, H. Pham, D. Sun, A. Smart, J. B. Schnermann, and J. P. Briggs. 1998. Regulation of cyclooxygenase expression in the kidney by dietary salt intake. *Am. J. Physiol.* **274:** F481–F489.
13. Burg, M. B., J. D. Ferraris, and N. I. Dmitrieva. 2007. Cellular response to hyperosmotic stresses. *Physiol. Rev.* **87:** 1441–1474.
14. Ho, S. N. 2003. The role of NFAT5/TonEBP in establishing an optimal intracellular environment. *Arch. Biochem. Biophys.* **413:** 151–157.
15. Woo, S. K., S. D. Lee, and H. M. Kwon. 2002. TonEBP transcriptional activator in the cellular response to increased osmolality. *Pflugers Arch.* **444:** 579–585.
16. Fagone, P., R. Sriburi, C. Ward-Chapman, M. Frank, J. Wang, C. Gunter, J. W. Brewer, and S. Jackowski. 2007. Phospholipid biosynthesis program underlying membrane expansion during B-lymphocyte differentiation. *J. Biol. Chem.* **282:** 7591–7605.
17. Tian, Y., R. Zhou, J. E. Rehg, and S. Jackowski. 2007. Role of phosphocholine cytidylyltransferase alpha in lung development. *Mol. Cell. Biol.* **27:** 975–982.
18. Jackowski, S. 1996. Cell cycle regulation of membrane phospholipid metabolism. *J. Biol. Chem.* **271:** 20219–20222.
19. Carter, J. M., K. A. Waite, R. B. Campenot, J. E. Vance, and D. E. Vance. 2003. Enhanced expression and activation of CTP:phosphocholine cytidylyltransferase beta2 during neurite outgrowth. *J. Biol. Chem.* **278:** 44988–44994.
20. van Meer, G., and K. Simons. 1986. The function of tight junctions in maintaining differences in lipid composition between the apical and the basolateral cell surface domains of MDCK cells. *EMBO J.* **5:** 1455–1464.
21. Fagone, P., and S. Jackowski. 2009. Membrane phospholipid synthesis and endoplasmic reticulum function. *J. Lipid Res.* **50 (Suppl.):** 311–316.
22. Hermansson, M., K. Hokynar, and P. Somerharju. 2011. Mechanisms of glycerophospholipid homeostasis in mammalian cells. *Prog. Lipid Res.* **50:** 240–257.
23. Singer, S. J., and G. L. Nicolson. 1972. The fluid mosaic model of the structure of cell membranes. *Science.* **175:** 720–731.
24. Sterin-Speziale, N., V. L. Kahane, C. P. Setton, M. C. Fernandez, and E. H. Speziale. 1992. Compartmental study of rat renal phospholipid metabolism. *Lipids.* **27:** 10–14.
25. Fernández-Tomé, M. C., and N. B. Sterin-Speziale. 1994. Short- and long-term treatment with indometacin causes renal phospholipid alteration: a possible explanation for indomethacin nephrotoxicity. *Pharmacology.* **48:** 341–348.
26. Setton-Avruj, C. P., M. D. Fernández-Tomé, A. Negri, A. Scerbo, E. Arrizurieta, and N. B. Sterin-Speziale. 1996. Is the increase in renal papillary phospholipid biosynthesis a protective mechanism against injury? *Kidney Blood Press. Res.* **19:** 38–45.
27. Setton-Avruj, C. P., and N. B. Sterin-Speziale. 1996. Renal phospholipid metabolism in streptozotocin-induced diabetic rats. *Kidney Blood Press. Res.* **19:** 128–135.

28. Herzlinger, D. A., T. G. Easton, and G. K. Ojakian. 1982. The MDCK epithelial cell line expresses a cell surface antigen of the kidney distal tubule. *J. Cell Biol.* **93:** 269–277.
29. Rindler, M. J., L. M. Chuman, L. Shaffer, and M. H. Saier, Jr. 1979. Retention of differentiated properties in an established dog kidney epithelial cell line (MDCK). *J. Cell Biol.* **81:** 635–648.
30. Niimura, Y., T. Moue, N. Takahashi, and K. Nagai. 2010. Medium osmolarity-dependent biosynthesis of renal cellular sulfoglycolipids is mediated by the MAPK signaling pathway. *Biochim. Biophys. Acta.* **1801:** 1155–1162.
31. Niimura, Y., T. Moue, N. Takahashi, and K. Nagai. 2010. Modification of sphingoglycolipids and sulfolipids in kidney cell lines under heat stress: activation of monohexosylceramide synthesis as a ceramide scavenger. *Glycobiology.* **20:** 710–717.
32. Pescio, L. G., N. O. Favale, M. G. Marquez, and N. B. Sterin-Speziale. 2012. Glycosphingolipid synthesis is essential for MDCK cell differentiation. *Biochim. Biophys. Acta.* **1821:** 884–894.
33. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193:** 265–275.
34. Michea, L., D. R. Ferguson, E. M. Peters, P. M. Andrews, M. R. Kirby, and M. B. Burg. 2000. Cell cycle delay and apoptosis are induced by high salt and urea in renal medullary cells. *Am. J. Physiol. Renal Physiol.* **278:** F209–F218.
35. Bligh, E. G., and W. J. Dyer. 1959. A rapid method of total lipid extraction and purification. *Can. J. Biochem. Physiol.* **37:** 911–917.
36. Bartlett, G. R. 1959. Phosphorus assay in column chromatography. *J. Biol. Chem.* **234:** 466–468.
37. Na, K. Y., S. K. Woo, S. D. Lee, and H. M. Kwon. 2003. Silencing of TonEBP/NFAT5 transcriptional activator by RNA interference. *J. Am. Soc. Nephrol.* **14:** 283–288.
38. Gekle, M., S. Wunsch, H. Oberleithner, and S. Silbernagl. 1994. Characterization of two MDCK-cell subtypes as a model system to study principal cell and intercalated cell properties. *Pflugers Arch.* **428:** 157–162.
39. Kwon, E. D., K. Zablocki, K. Y. Jung, E. M. Peters, A. Garcia-Perez, and M. B. Burg. 1995. Osmoregulation of GPC:choline phosphodiesterase in MDCK cells: different effects of urea and NaCl. *Am. J. Physiol.* **269:** C35–C41.
40. Nakanishi, T., R. S. Balaban, and M. B. Burg. 1988. Survey of osmolytes in renal cell lines. *Am. J. Physiol.* **255:** C181–C191.
41. Dmitrieva, N., D. Kultz, L. Michea, J. Ferraris, and M. Burg. 2000. Protection of renal inner medullary epithelial cells from apoptosis by hypertonic stress-induced p53 activation. *J. Biol. Chem.* **275:** 18243–18247.
42. Zhang, Z., N. I. Dmitrieva, J. H. Park, R. L. Levine, and M. B. Burg. 2004. High urea and NaCl carbonylate proteins in renal cells in culture and in vivo, and high urea causes 8-oxoguanine lesions in their DNA. *Proc. Natl. Acad. Sci. USA.* **101:** 9491–9496.
43. Alfieri, R. R., and P. G. Petronini. 2007. Hyperosmotic stress response: comparison with other cellular stresses. *Pflugers Arch.* **454:** 173–185.
44. Harris, R. C. 2006. COX-2 and the kidney. *J. Cardiovasc. Pharmacol.* **47 (Suppl.):** 37–42.
45. Fernández-Tomé, M., L. Kraemer, S. C. Federman, N. Favale, E. Speziale, and N. Sterin-Speziale. 2004. COX-2-mediated PGD2 synthesis regulates phosphatidylcholine biosynthesis in rat renal papillary tissue. *Biochem. Pharmacol.* **67:** 245–254.
46. Fernández-Tomé, M. C., E. H. Speziale, and N. B. Sterin-Speziale. 1997. Endogenous prostaglandins regulate rat renal phospholipid 'de novo' synthesis. *Biochim. Biophys. Acta.* **1349:** 55–66.
47. Dmitrieva, N. I., L. F. Michea, G. M. Rocha, and M. B. Burg. 2001. Cell cycle delay and apoptosis in response to osmotic stress. *Comp. Biochem. Physiol. A Mol. Integr. Physiol.* **130:** 411–420.
48. Rodriguez-Boulan, E., G. Kreitzer, and A. Musch. 2005. Organization of vesicular trafficking in epithelia. *Nat. Rev. Mol. Cell Biol.* **6:** 233–247.
49. Lykidis, A., and S. Jackowski. 2001. Regulation of mammalian cell membrane biosynthesis. *Prog. Nucleic Acid Res. Mol. Biol.* **65:** 361–393.
50. Tian, W., Z. Zhang, and D. M. Cohen. 2000. MAPK signaling and the kidney. *Am. J. Physiol. Renal Physiol.* **279:** F593–F604.
51. Sheikh-Hamad, D., and M. C. Gustin. 2004. MAP kinases and the adaptive response to hypertonicity: functional preservation from yeast to mammals. *Am. J. Physiol. Renal Physiol.* **287:** F1102–F1110.
52. Fernández-Tomé, M., N. Favale, L. Kraemer, M. Gabriela Marquez, E. Speziale, and N. Sterin-Speziale. 2004. p44/42(ERK1/2) MAPK and PLD activation by PGD2 preserves papillary phosphatidylcholine homeostasis. *Biochem. Biophys. Res. Commun.* **320:** 1055–1062.
53. Kennedy, E. P. 1959. The enzymic synthesis of phospholipids. *Prog. Neurobiol.* **4:** 260–264.
54. Yeagle, P. L. 1989. Lipid regulation of cell membrane structure and function. *FASEB J.* **3:** 1833–1842.
55. Lee, A. G. 2004. How lipids affect the activities of integral membrane proteins. *Biochim. Biophys. Acta.* **1666:** 62–87.
56. Brenner, R. R. 1984. Effect of unsaturated acids on membrane structure and enzyme kinetics. *Prog. Lipid Res.* **23:** 69–96.
57. Liscovitch, M., and L. C. Cantley. 1994. Lipid second messengers. *Cell.* **77:** 329–334.
58. Kwon, H. M., A. Yamauchi, S. Uchida, R. B. Robey, A. Garcia-Perez, M. B. Burg, and J. S. Handler. 1991. Renal Na-myo-inositol cotransporter mRNA expression in Xenopus oocytes: regulation by hypertonicity. *Am. J. Physiol.* **260:** F258–F263.
59. Favale, N. O., N. B. Sterin Speziale, and M. C. Fernandez Tome. 2007. Hypertonic-induced lamin A/C synthesis and distribution to nucleoplasmic speckles is mediated by TonEBP/NFAT5 transcriptional activator. *Biochem. Biophys. Res. Commun.* **364:** 443–449.
60. van Meer, G., E. H. Stelzer, R. W. Wijnaendts-van-Resandt, and K. Simons. 1987. Sorting of sphingolipids in epithelial (Madin-Darby canine kidney) cells. *J. Cell Biol.* **105:** 1623–1635.
61. Meder, D., A. Shevchenko, K. Simons, and J. Fullekrug. 2005. Gp135/podocalyxin and NHERF-2 participate in the formation of a preapical domain during polarization of MDCK cells. *J. Cell Biol.* **168:** 303–313.
62. Kent, C. 1995. Eukaryotic phospholipid biosynthesis. *Annu. Rev. Biochem.* **64:** 315–343.
63. Kwon, E. D., K. Y. Jung, L. C. Edsall, H. Y. Kim, A. Garcia-Perez, and M. B. Burg. 1995. Osmotic regulation of synthesis of glycerophosphocholine from phosphatidylcholine in MDCK cells. *Am. J. Physiol.* **268:** C402–C412.
64. Fagone, P., and S. Jackowski. Phosphatidylcholine and the CDP-choline cycle. *Biochim. Biophys. Acta.* Epub ahead of print. September 23, 2012; doi:pii: S1388-1981.