Cellular/Molecular

PLCδ1 Plays Central Roles in the Osmotic Activation of ΔN-TRPV1 Channels in Mouse Supraoptic Neurons and in Murine Osmoregulation

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The magnocellular neurosecretory cells (MNCs) of the hypothalamus play a vital role in osmoregulation, but the mechanisms underlying MNC osmosensitivity are not fully understood. We showed previously that high osmolality activates phospholipase C (PLC) in rat MNCs in a Ca^{2+}-dependent manner and that PLC activation is necessary for full osmotic activation of an N-terminal variant of the TRPV1 (ΔN-TRPV1) channel. We therefore hypothesized that the Ca^{2+}-dependent δ1 isoform of PLC contributes to ΔN-TRPV1 activation and tested whether MNC function is defective in a transgenic PLCδ1 KO mouse. Water deprivation for 24 h caused greater increases in serum osmolality and losses in body weight in PLCδ1 KO mice than it did in control mice. Action potentials and ΔN-TRPV1 currents were measured in acutely isolated mouse MNCs using whole-cell patch clamp before and after exposure to hypertonic solutions. This treatment elicited a significant activation of ΔN-TRPV1 currents and an increase in firing rate in MNCs isolated from control mice, but not from PLCδ1 KO mice. Submembranous filamentous actin was measured in isolated MNCs before and after treatment with angiotensin II and hypertonic solution. Both treatments caused an increase in filamentous actin fluorescence in MNCs isolated from control mice, but both responses were significantly attenuated in MNCs from PLCδ1 KO mice. Our data demonstrate that the PLCδ1 isoform plays a key role in the activation of ΔN-TRPV1 channels and in osmosensory transduction in MNCs. This study advances our understanding of the molecular mechanisms underlying mammalian osmoregulation.

Key words: **osmoregulation; osmosensitivity; phospholipase C; supraoptic nucleus; TRPV1**

Significance Statement

Magnocellular neurosecretory cells (MNCs) of the hypothalamus play a central role in osmoregulation. We have identified a key role for the PLCδ1 isoform in the activation of ΔN-TRPV1 channels and osmosensory transduction in MNCs. The data indicate that the PLCδ1 isoform is activated by the Ca^{2+} influx occurring during MNC action potentials and exerts a positive feedback on ΔN-TRPV1 channels to enhance MNC excitability. This study provides evidence that PLCδ1 is a key molecule underlying osmosensory transduction, the regulation of VP release, and osmoregulation.

Introduction

Osmoregulation is an essential homeostatic process. Mammals use robust regulatory mechanisms to maintain the osmolality of their body fluids within very narrow limits; and exceeding these limits can lead to serious deleterious consequences, largely through osmotically induced shrinkage and swelling of brain tissue (Bourque, 2008).

The most important regulator of water excretion is the hormone vasopressin (VP), which acts on the kidney to enhance water retention. VP is released by the magnocellular neurosecretory cells of the hypothalamus (MNCs), which are "osmosensitive" in that their electrical activity depends on the osmolality of the extracellular fluid. MNCs rapidly increase their firing rate when the external osmolality is

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increased, leading to an increase in VP release (Bourque et al., 1994; Oliet and Bourque, 1994).

MNCs lack the acute volume regulatory mechanisms found in many central neurons, and this enables them to transduce osmotically induced changes in cell volume into electrical signals (Zhang and Bourque, 2003). This is accomplished through regulation of a mechanically activated nonselective cation current mediated by an N-terminal variant of the TRPV1 channel (ΔN-TRPV1) (Oliet and Bourque, 1993; Sharif-Naeini et al., 2006) that is activated by osmotically induced cell shrinkage. These channels depolarize the MNCs and make them more likely to fire action potentials in response to excitatory inputs from other osmosensitive neurons (Sudbury et al., 2010; Prager-Khoutorsky and Bourque, 2015).

The osmotic activation of ΔN-TRPV1 occurs in a cytoskeleton-dependent fashion involving both cortical actin and microtubules (Zhang et al., 2007; Prager-Khoutorsky and Bourque, 2015). The polymerization of filamentous actin (F-actin) enhances the gating of ΔN-TRPV1 channels in MNCs (Zhang et al., 2007), possibly through regulating the mechanical support of the membrane (Prager-Khoutorsky, 2017). The MNC cytoskeleton and its relationship with ΔN-TRPV1 channels are regulated by phospholipase C (PLC). Angiotensin II (Ang II) excites MNCs (Yang et al., 1992) by amplifying mechanosensory transduction through a PLC- and protein kinase C (PKC)-dependent increase in F-actin density (Yang et al., 1992; Zhang and Bourque, 2008; Prager-Khoutorsky and Bourque, 2010).

PLC is activated by increases in osmolality, and this activation is dependent on Ca^{2+} influx through L-type Ca^{2+} channels (Shah et al., 2014). The osmotic activation of ΔN-TRPV1 current is suppressed by the presence of PLC antagonists, suggesting that PLC activation is necessary for its full activation (Bansal and Fisher, 2017). These data suggest that mechanical activation of ΔN-TRPV1 leads to MNC depolarization, an increase in action potential firing, and Ca^{2+} influx through low- and high-voltage-activated L-type Ca^{2+} channels (Fisher and Bourque, 1995). The resultant increase in intracellular Ca^{2+} activates PLC to cause a positive feedback loop, leading to a stronger activation of ΔN-TRPV1, greater action potential firing, and enhanced release of VP. PLC may therefore play a key role in MNC osmosensitivity. The Ca^{2+} dependence of the activation of PLC led us to hypothesize that the responsible isoform is a member of the PLCδ family, whose members are activated primarily by intracellular Ca^{2+} (Essen et al., 1996). PLCδ isoforms are widely expressed in the CNS, but their role in neuronal function is not well characterized (Kadamur and Ross, 2013; Nakamura and Fukami, 2017). PLCδ1 is expressed in almost all regions of the mouse brain and is the most Ca^{2+}-sensitive isoform in the PLCδ family (Suh et al., 1988; Lee et al., 1999). MNCs have been shown to express PLCδ1 (Hazell et al., 2012), and we therefore used transgenic mice in which this isoform has been knocked out to test whether and how it is involved in MNC osmosensitivity (Fukami et al., 2001; Nakamura et al., 2003; Hazell et al., 2012).

We report here that PLCδ1 KO mice display a marked defect in their ability to respond to an osmotic challenge and that MNCs isolated from PLCδ1 KO mice display a defective electrophysiological response to increases in external osmolality. These data suggest that the PLCδ1 isoform is activated by the Ca^{2+} influx occurring during osmotically evoked MNC action potential firing and exerts a positive feedback on ΔN-TRPV1 channels to enhance MNC excitability. We further show that MNCs isolated from PLCδ1 KO mice display defective osmotic regulation of F-actin, suggesting that this defect may contribute to the decreased osmotic activation of ΔN-TRPV1 channels. PLCδ1 may therefore be a key component of the machinery underlying osmosensory transduction and osmoregulation of VP release in MNCs.

Materials and Methods

Ethical approval. This work was approved by the University of Saskatchewan's Animal Research Ethics Board and adhered to the Canadian Council on Animal Care guidelines for humane animal use.

Animals. C57BL/6J mice (control) were purchased from the Charles River Laboratories at 6-8 weeks of age. Homozygous PLCδ1 KO mice were bred at 6-8 weeks of age. Upon delivery to our animal facility, mice were individually housed under identical conditions with a standard light-dark cycle for 2-4 weeks. The experiments were performed from mice at 8-14 weeks of age. We used PLCδ1 KO mice of both sexes for the osmolality experiments, and both males ($N = 22$) and females ($N = 12$) showed a response to water deprivation that was significantly greater than did control mice ($p < 0.01$ for both comparisons). Only male mice were used for the electrophysiological and F-actin experiments.

Isolation of MNCs. MNCs were isolated as previously described (Bansal and Fisher, 2017). Neurons with a maximal cross-sectional area >160 μm^2 were identified as MNCs, which is a criterion that is valid for both rat (Oliet and Bourque, 1992) and mouse MNCs (Sharif-Naeini et al., 2008). We did not differentiate between VP and oxytocin-expressing MNCs, as both types have been shown to be sensitive to changes in osmolality (Bourque and Oliet, 1997). Isolation of MNCs using a similar method resulted in isolated MNCs that were mostly (~80%) VP expressing (Sharif-Naeini et al., 2008).

Solutions. The isotonic extracellular solution contained the following (in mM): 135 NaCl, 5 KCl, 1 $MgCl_2$, 2 $CaCl_2$, 10 glucose, 10 HEPES, pH 7.4, by NaOH. The intracellular solution contained the following (in mM): 125 KCl, 10 HEPES, 1 $MgCl_2$, 4 Na_2-ATP, 1 Na-GTP, 14 Tris-phosphocreatine, 0.5 EGTA, pH adjusted to 7.2 by KOH. The osmolalities of solutions were adjusted with mannitol to 310 (external) and 280 mosmol kg^{-1} (internal), respectively. The hypertonic external solution was adjusted to 340 mosmol kg^{-1} by the addition of further mannitol.

Measurements of serum osmolality and body weight. Serum osmolality and body weight were measured in control and PLCδ1 KO mice under normal hydration (i.e., with free access to water) or after 24 h of water deprivation. Mice were killed, and the blood samples collected, at the same time each day. Blood samples were centrifuged (2000 rpm, 2 min), the serum was collected, and its osmolality was measured with an osmometer (Wescor, VaporPressure Osmometer model 5520). The measured serum osmolality of PLCδ1 KO mice before water deprivation (323.4 ± 12.0 mosmol kg^{-1}; $N = 34$) was not significantly different from that of control mice (317.3 ± 5.1 mosmol kg^{-1}; $N = 15$; $p > 0.05$; see Fig. 1*A*). Body weight was measured with a scale (Ohaus, CS200) at the same time each day. The mean body weight of the PLCδ1 KO mice (26.7 ± 2.4 g; $N = 13$) before the water deprivation was slightly but significantly higher than that of the control mice (24.0 ± 1.6 g; $N = 10$).

Patch-clamp recording. Whole-cell patch-clamp recordings were performed 2-4 h after cell isolation. Glass microelectrodes (1.5 × 0.86 mm, Sutter Borosilicate Glass) were pulled with a P-97 horizontal pipette puller (Sutter Instrument). The pipette resistance was between 2.5 and 3.5 MΩ. Signals were amplified by using an EPC-9 amplifier (HEKA Elektronik). Series resistance was compensated by 75%-80% using the amplifier circuitry. In voltage-clamp experiments, cells were clamped at −60 mV and then ramp voltage commands (−100 to −20 mV, 5 s, 1 kHz sampling) were applied. Junction potentials were corrected on recording. In whole-cell current-clamp experiments, the holding current was initially adjusted to achieve a membrane potential of ~−60 mV to allow the cell to stabilize for 3 min before determining the firing rate. The signal was sampled at 1 kHz, and the frequency of action potentials was determined over 50 s. Measurements of firing frequency were taken 3 min after the addition of hypertonic solution. Recordings were analyzed using Clampfit 10.0 (Molecular Devices), GraphPad Prism 6.01, and Origin 8.1 (OriginLab).

F-actin staining. Acutely isolated MNCs from control and PLCδ1 KO mice were subjected to F-actin staining with Texas Red-X Phalloidin (Invitrogen) using a protocol that has been previously described (Zhang and Bourque, 2008). Images were obtained using an LSM700 laser scanning confocal microscope (Carl Zeiss), and *Z*-stack images were collected for cells deemed to be MNCs. All captured images were analyzed using ImageJ software (National Institutes of Health) by tracing the perimeter of each MNC by following the line of greatest fluorescence and determining the mean fluorescence of pixels on that line. Negative control experiments in which the Texas Red-X Phalloidin was excluded showed no significant staining of the MNC plasma membrane.

Drugs. All drugs were prepared as a stock solution and diluted to their final concentrations in bath solution. Drugs were dissolved in distilled water or DMSO. Ang II (A9525), Triton X-100 (X100), donkey serum (D9663), and sodium azide (438456) were purchased from Sigma Millipore.

Statistical analysis. All data are expressed as mean ± SD. *N* and *n* were used to indicate the number of animals and cells, respectively. Significant differences were determined by the Student's *t* test, one-way ANOVA, or the Fisher's exact test as appropriate. A value of $p < 0.05$ was considered statistically significant.

Results

Impaired osmoregulation in response to water deprivation in PLCδ1 KO mice

The effect of 24 h of water deprivation on serum osmolality and body weight was examined in control and PLCδ1 KO mice. Serum osmolality was significantly higher following dehydration in both control (from 317.3 ± 5.1, $N = 15$, to 329.4 ± 4.5 mOsm, $N = 15$; $p < 0.01$) and PLCδ1 KO mice (from 323.4 ± 12.0, $N = 30$, to 352.2 ± 11.1 mOsm, $N = 34$; $p < 0.001$; Fig. 1*A*). The serum osmolality following water deprivation was, however, significantly higher in PLCδ1 KO mice ($p < 0.001$; Fig. 1*A*), suggesting that these mice exhibit dysfunctional osmoregulation. Serum osmolality before water deprivation was not significantly different between control and PLCδ1 KO mice ($p > 0.05$; Fig. 1*A*). Water deprivation caused a loss of body weight in both control (2.9 ± 0.3 g; $N = 10$) and PLCδ1 KO mice (3.8 ± 0.4 g; $N = 10$), but the loss of weight was significantly greater in PLCδ1 KO mice ($p < 0.01$). Figure 1*B* shows the loss of weight in the two strains of mice as a percentage of original body weight. Control mice lost 12.2 ± 1.1% of body weight ($N = 10$), whereas PLCδ1 KO mice lost 13.9 ± 1.1% ($N = 10$), which is a significantly greater percentage ($p < 0.01$). These data support the hypothesis that osmoregulation is defective in PLCδ1 KO mice.

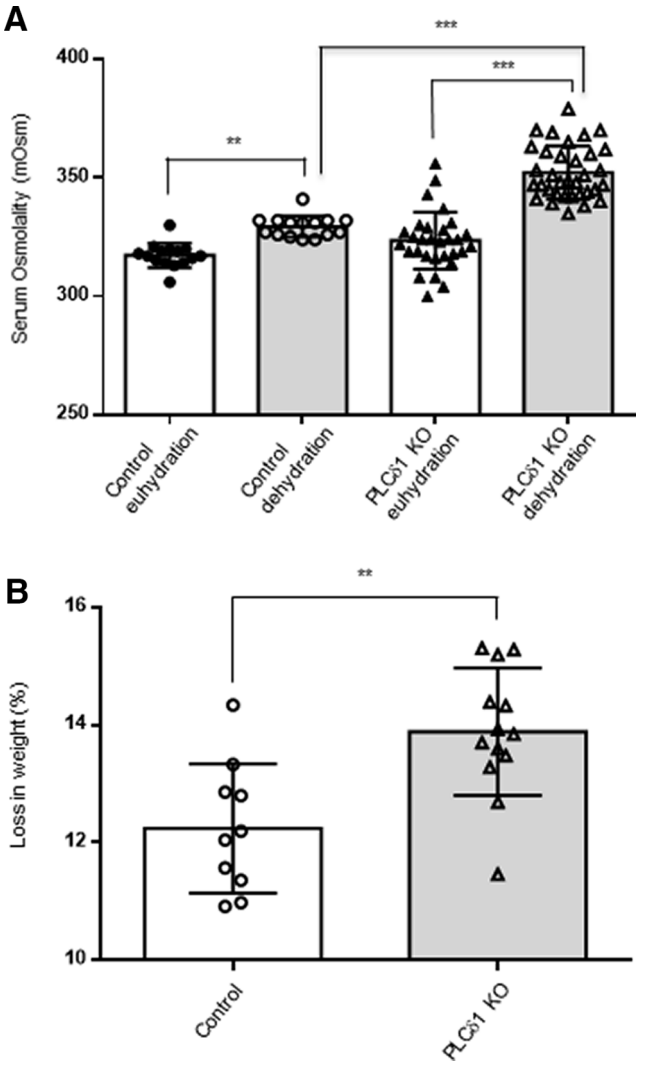

Figure 1. The effect of water deprivation on serum osmolality and weight loss in control and PLCδ1 KO mice. ***A***, Bar-scatter plot illustrating the serum osmolality following 24 h of water deprivation in control and PLCδ1 KO MNC. The increase of serum osmolality by dehydration was significantly higher in PLCδ1 KO mice (352.2 ± 11.1 mosmol kg^{-1}; $N = 34$) than in control mice (329.4 ± 4.5 mosmol kg^{-1}; control; $N = 15$; Bonferroni's *post hoc* test; $p < 0.001$). ***B***, Bar-scatter plot illustrating the weight loss on water deprivation for 24 h in control and PLCδ1 KO MNC. The percentage of weight loss was significantly higher in PLCδ1 KO mice (13.9 ± 1.1%; $N = 13$) compared with control mice (12.2 ± 1.1%; $N = 10$; Student *t* test; $p < 0.01$). $^{**}p < 0.01$. $^{***}p < 0.001$.

Hyperosmotic challenge elicits an increase in action potential firing in MNCs from control but not PLCδ1 KO mice

The above data suggest that the osmoregulatory system in PLCδ1 KO mice fails to respond appropriately when the animal experiences increased osmolality. Our previous data suggest that PLC plays a role in the osmosensitivity of MNCs, and we therefore hypothesized that MNCs isolated from PLCδ1 KO mice would fail to respond normally when exposed to hypertonic solutions. We first measured action potential firing in single MNCs isolated from control and PLCδ1 KO mice under whole-cell current clamp in either isotonic or hypertonic solution (Fig. 2*A*). In MNCs from control mice, the rate of firing in isotonic solution was 0.21 ± 0.16 Hz (iso, $n = 16$) and was significantly higher in hypertonic solution at 0.36 ± 0.17 Hz (hyper, $n = 15$; $p < 0.05$; Fig. 2*B*). The firing rates of isolated MNCs are lower than those of MNCs *in situ*, and these results are comparable to a previous study showing the effects of hypertonic solution on the firing of isolated MNCs (Sharif-Naeini et al., 2008). In MNCs isolated from PLCδ1 KO mice, however, there was no significant difference between the rates of firing in isotonic solution (0.22 ± 0.10 Hz; iso; $n = 14$) and hypertonic solution (0.24 ± 0.15 Hz; hyper, $n = 12$; $p > 0.05$; Fig. 2*B*). Analysis of single action potentials (Fig. 2*A*, insets) revealed that the maximum velocity of action potential depolarization (rising phase) determined from the first derivative of the action potential waveform was significantly increased by hypertonic solution in MNCs isolated from control mice (27.1 ± 12.8 $mVms^{-1}$; iso, $n = 13$, to 42.1 ± 14.8 $mVms^{-1}$; hyper, $n = 14$; $p < 0.05$), but not in MNCs isolated from PLCδ1 KO mice (27.7 ± 9.5 $mVms^{-1}$; iso, $n = 12$, to 31.3 ± 5.6 $mVms^{-1}$; hyper, $n = 9$; $p > 0.05$; Fig. 2*C*). The maximum velocities of action potential hyperpolarization (i.e., the decaying phase) were not significantly changed in MNCs isolated from control mice (−19.2 ± 5.7 $mVms^{-1}$; iso, $n = 13$, to −17.9 ± 12.6 $mVms^{-1}$; hyper, $n = 14$; $p > 0.05$) or MNCs isolated from

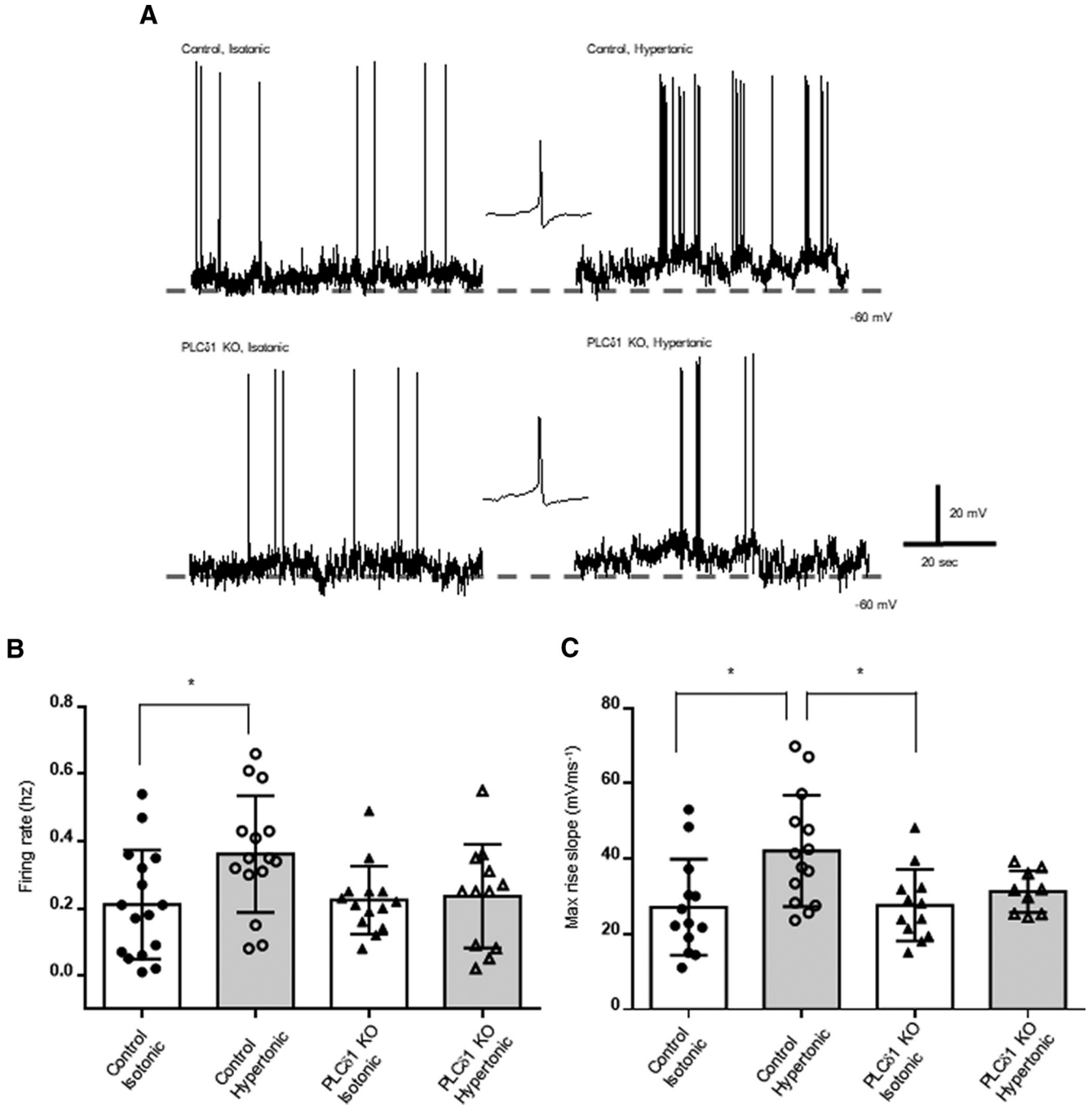

Figure 2. The effect of hypertonic solution on action potential firing in MNCs isolated from control and PLCδ1 KO mice. ***A***, Representative current-clamp recordings from MNCs showing action potential firing after exposure to isotonic (left) and hypertonic solutions (right) for 10 min in control (top) and PLCδ1 KO mice (bottom). Representative single action potentials are highlighted (insets). ***B***, Bar-scatter plot illustrating firing rates in MNCs from control and PLCδ1 KO mice. The firing rate was significantly higher in hypertonic solution in MNCs isolated from control (the rate increased from 0.21 ± 0.16 Hz; iso; $n = 16$, to 0.36 ± 0.17 Hz; hyper, $n = 15$; Bonferroni's *post hoc* test, $p < 0.05$) but not PLCδ1 KO mice (the rate in isotonic solution 0.22 ± 0.10 Hz; iso; $n = 14$, was not different from that in hypertonic solution 0.24 ± 0.15 Hz; hyper, $n = 12$; $p > 0.05$). ***C***, Bar-scatter plot illustrating the maximum rising velocity of single action potential in MNCs isolated from control and PLCδ1 KO mice. The maximum rising velocity was significantly greater in hypertonic solution in MNCs isolated from control (the rising velocity increased from 27.1 ± 12.8 $mVms^{-1}$; isotonic, $n = 13$, to 42.1 ± 14.8 $mVms^{-1}$; hyper, $n = 14$; Bonferroni's *post hoc* test, $p < 0.05$) but not in MNCs isolated from PLCδ1 KO mice (27.7 ± 9.5 $mVms^{-1}$; isotonic, $n = 12$, and 31.3 ± 5.6 $mVms^{-1}$; hypertonic, $n = 9$; $p > 0.05$). *$p < 0.05$.

PLCδ1 KO mice (−15.7 ± 4.3 $mVms^{-1}$; iso, $n = 14$, to −17.5 ± 1.6 $mVms^{-1}$; hyper, $n = 12$; $p > 0.05$; data not shown). There was also a significant difference in the depolarization caused by hypertonic solution in MNCs isolated from the two strains of mice. Hypertonic solutions caused a significant depolarization in MNCs from control mice (from −58.5 ± 6.3 mV; $n = 16$ to −53.3 ± 3.1 mV; $n = 15$; $p < 0.05$) but did not cause a significant depolarization in MNCs from PLCδ1 KO mice (from −56.9 ± 6.4 mV; $n = 14$ to −55.5 ± 4.4 mV; $n = 12$; $p > 0.05$).

PLCδ1 KO mice exhibit attenuated ΔN-TRPV1-mediated cation currents in response to hypertonicity and Ang II

The activation of ΔN-TRPV1 cation channels is a central component of the osmotic activation of MNCs (Bourque, 2008; Zaelzer

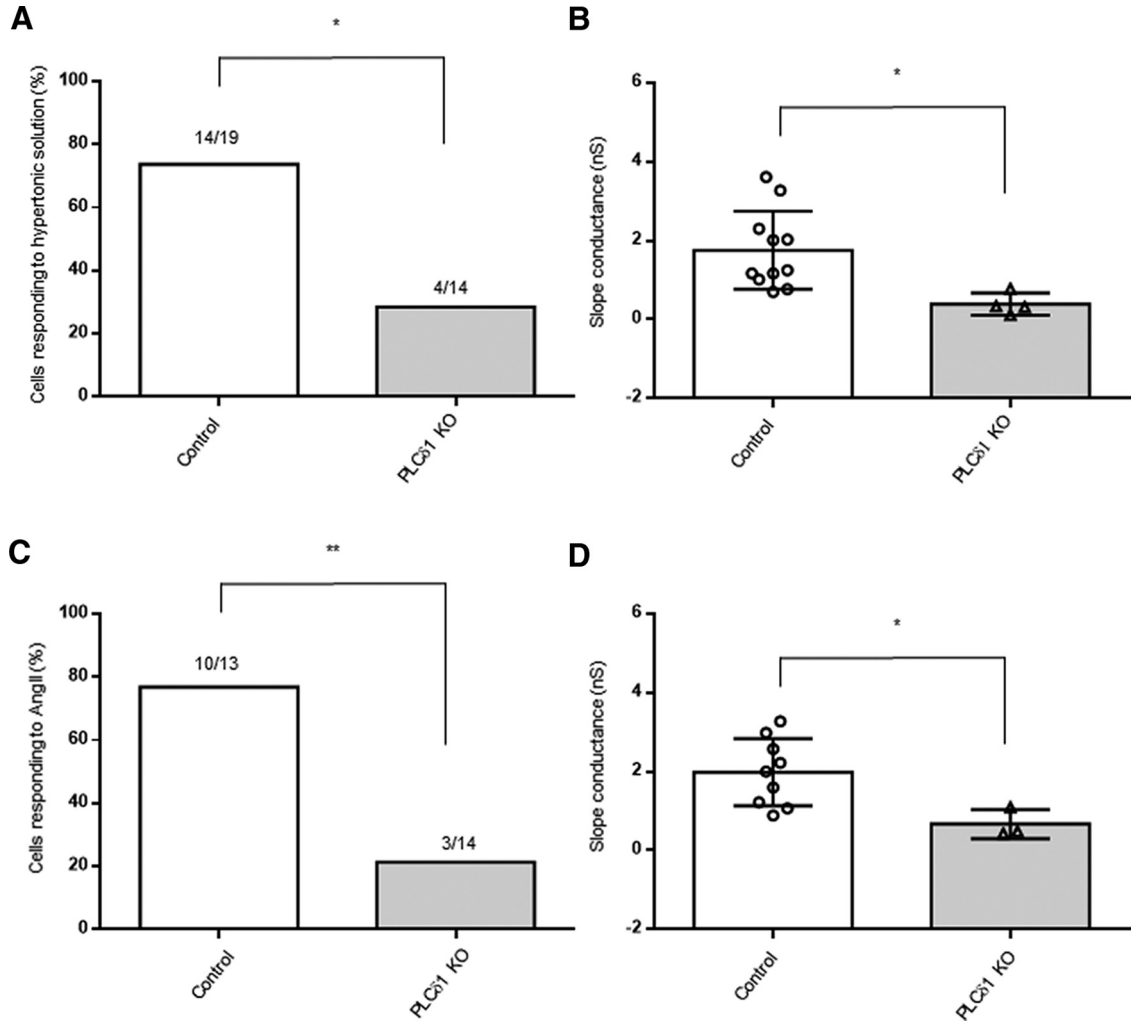

Figure 3. The activation of ΔN-TRPV1 currents in MNCs isolated from control and PLCδ1 KO mice in response to hypertonic solution and Ang II. ***A***, Bar graph illustrating the proportion of MNCs from control and PLCδ1 KO mice responding to hypertonic solution. The proportion responding was significantly reduced in MNCs isolated from PLCδ1 KO mice (control; 14 of 19, 73.7% vs PLCδ1 KO; 4 of 14, 28.6%, Fisher's exact test, $p < 0.05$). ***B***, Bar-scatter plot illustrating the amplitude of the current (expressed as slope conductance in nS) evoked in response to hypertonic solution in MNCs isolated from responding MNCs isolated from control and PLCδ1 KO mice. The conductance evoked was significantly reduced in MNCs from PLCδ1 KO mice (control; 1.76 ± 0.99 nS, $n = 11$ vs PLCδ1 KO; 0.39 ± 0.28 nS, $n = 4$, unpaired t test, $p < 0.05$). ***C***, Bar graph illustrating the proportion of MNCs from control and PLCδ1 KO mice responding to Ang II. The proportion responding was significantly reduced in MNCs isolated from PLCδ1 KO mice (control: 10 of 13, 76.9%, PLCδ1 KO: 3 of 14, 21.4%, Fisher's exact test, $p < 0.01$). ***D***, Bar-scatter plot illustrating the amplitude of the current (expressed as slope conductance in nS) evoked in response to Ang II in MNCs isolated from control and PLCδ1 KO mice. The conductance evoked was significantly reduced in MNCs from PLCδ1 KO mice (control; 1.98 ± 0.85 nS, $n = 9$ vs PLCδ1 KO; 0.67 ± 0.37 nS, $n = 3$, unpaired t test, $p < 0.05$). *$p < 0.05$. **$p < 0.01$.

et al., 2015), and our laboratory has shown that PLC contributes to this activation in isolated rat MNCs (Bansal and Fisher, 2017). We therefore measured the osmotic activation of ΔN-TRPV1 cation currents in MNCs from control and PLCδ1 KO mice using a ramp protocol under whole-cell patch clamp (Oliet and Bourque, 1993; Bansal and Fisher, 2017). Ramp pulses were applied in isotonic solution and then again 2 min after administration of hypertonic solution. MNCs showing a change in slope conductance <0.01 nS were considered nonresponsive. The application of hypertonic solution increased a nonselective cation current in 73.7% of MNCs (14 of 19 tested) from control mice but in only 28.6% (4 of 14 MNCs tested) of MNCs isolated from PLCδ1 KO mice, which is a significantly lower proportion (Fig. 3*A*; $p < 0.05$). The slope conductance of osmotically evoked currents measured at −60 mV was, furthermore, significantly lower in MNCs from control mice (1.76 ± 0.99 nS, $n = 11$) compared with those in MNCs from PLCδ1 KO mice (0.39 ± 0.28 nS, $n = 4$; $p < 0.05$; Fig. 3*B*). The amplitude of currents evoked by hypertonic solution was therefore markedly reduced in MNCs isolated from PLCδ1 KO mice. We also tested the response of the two strains of mice to Ang II. Ang II has been shown to enhance the osmosensitivity of ΔN-TRPV1 channels in MNCs through a PLC-dependent mechanism (Zhang and Bourque, 2008) and to activate PLC in MNCs isolated from rat MNCs (Bansal and Fisher, 2017). We therefore tested the responses to Ang II in MNCs isolated from our two strains of mice to determine whether PLCδ1 may be involved in the response. Ramp voltage commands (from −100 to −20 mV over a period of 5 s) were applied in isotonic solution and then again 2 min after administration of Ang II (0.2 μM). MNCs showing a change in slope conductance <0.01 nS were considered nonresponsive. The proportion of MNCs that responded to Ang II was significantly reduced in PLCδ1 KO mice (3 of 14, 21.4%) compared with the proportion from control mice (10 of 13, 76.9%; $p < 0.01$; Fig. 3*C*). Furthermore, the slope conductance of the current evoked by Ang II was significantly decreased in MNCs isolated from PLCδ1 KO mice that responded (0.67 ± 0.37 nS, $n = 3$) compared with those from control mice (1.98 ± 0.85 nS, $n = 9$; $p < 0.05$; Fig. 3*D*).

Figure 4A (top traces) shows evoked ramp currents in a typical responding cell before and after the addition of hypertonic solution, and Figure 4A (bottom) shows the difference between the two traces (i.e., the current included by hypertonic solution). The mean reversal potential of hypertonic-sensitive currents was −41.6 ± 5.6 mV (n = 14), which is consistent with that observed previously in rat MNCs (Oliet and Bourque, 1993; Bansal and Fisher, 2017) and in mouse MNCs (Sharif-Naeini et al., 2008). We therefore tested whether activation of the current could be prevented by a TRPV1 antagonist. Traces obtained from a typical experiment are shown in Figure 4B. In the presence of 1 μM SB366791, there was no significant difference between the slope conductance at −60 mV in isotonic solution (1.77 ± 0.82; n = 11) and following administration of hypertonic solution (1.68 ± 0.87; n = 11; $p > 0.05$). This is consistent with what was seen previously in mice MNCs (Ciura et al., 2011) and supports the hypothesis that the osmotic current that we observed is mediated by ΔN-TRPV1, which is the only variant that was detected in most MNCs and the only variant that undergoes osmotic activation (Zaelzer et al., 2015). The response to hypertonic solution by MNCs isolated from PLCδ1 KO mice was dramatically reduced. Figure 4C (top traces) shows evoked ramp currents in a typical (nonresponding) MNC from a PLCδ1 KO mouse before and after the addition of hypertonic solution, and Figure 4C (bottom) shows the difference between the two traces (i.e., the current induced by hypertonic solution). In this MNC, no hypertonic current was induced. Figure 4D shows the currents in MNCs isolated from control and PLCδ1 KO mice induced by the administration of 0.2 μM Ang II. The response to Ang II in MNCs from PLCδ1 KO mice was dramatically reduced.

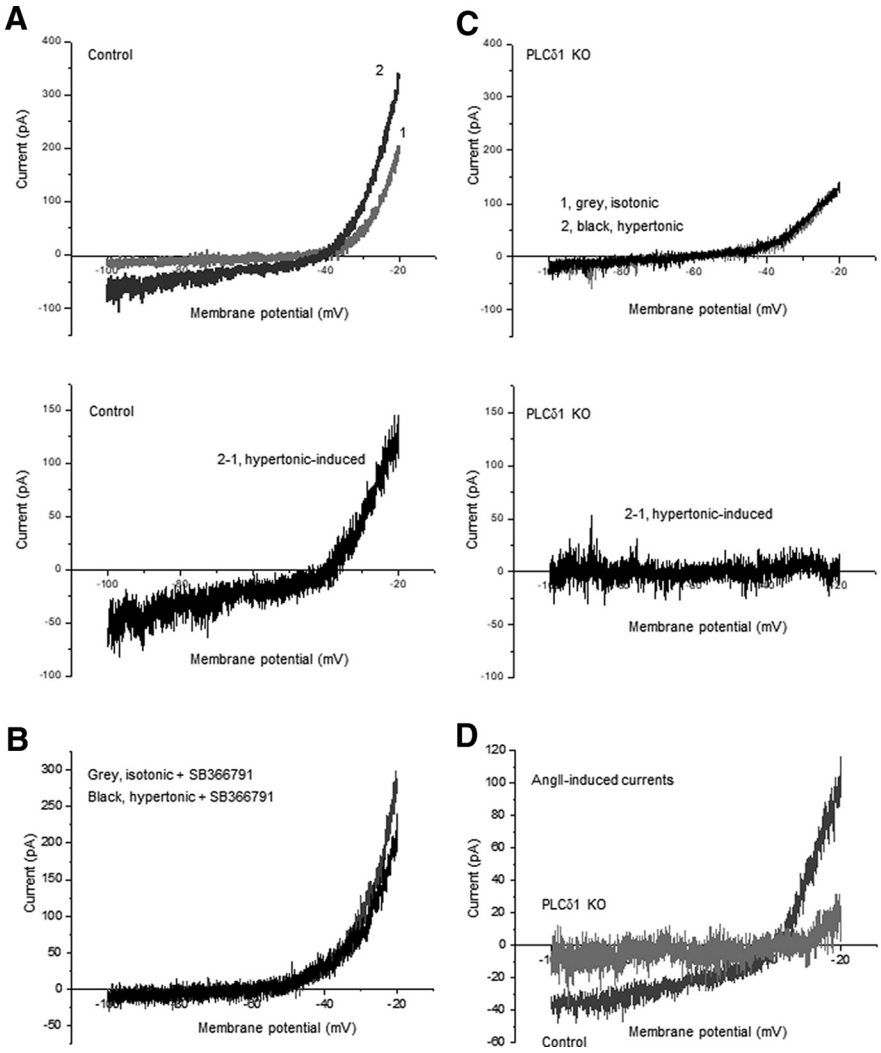

Figure 4. The osmotic activation of ΔN-TRPV1 cation currents in MNCs isolated from control and PLCδ1 KO mice. ***A***, Top, Representative current traces obtained in response to a ramp voltage applied to an MNC isolated from a control mouse in isotonic solution (gray; labeled 1) and following administration of hypertonic solution (black; labeled 2). Bottom, The current resulting from the subtraction of trace 2 from trace 1, which represents the hypertonic induced current. ***B***, Representative current traces obtained in response to a ramp voltage applied to an MNC isolated from a control mouse in isotonic solution (gray) and following administration of hypertonic solution (black) in the presence of 1 μM SB366791. ***C***, Top traces, Representative current traces obtained in response to a ramp voltage applied to an MNC isolated from a PLCδ1 KO mouse in isotonic solution (gray; labeled 1) and following administration of hypertonic solution (black; labeled 2). Bottom, The current resulting from the subtraction of trace 2 from trace 1, which represents the hypertonic induced current. ***D***, Representative traces showing currents evoked by the administration of Ang II (0.2 μM) in an MNC isolated from a control mouse (black) and in an MNC isolated from a PLCδ1 KO mouse (gray).

Our experiments suggest that the resting membrane potential (i.e., the potential at which zero current is being injected in our voltage-clamp experiments) would be depolarized by 17.3 ± 7.1 mV (n = 14) in the responding MNCs isolated from control mice but only 6.3 ± 1.2 mV (n = 4) in the responding MNCs isolated from PLCδ1 KO mice ($p < 0.01$). Although some MNCs isolated from PLCδ1 KO mice show some response to hypertonic solution, the response is greatly reduced compared with that observed in control mice.

F-actin did not increase in response to hypertonic solution and Ang II in PLCδ1 KO mice

The regulation of ΔN-TRPV1 activity by Ang II in MNCs has been shown to depend at least in part on PLC-dependent regulation of F-actin (Prager-Khoutorsky and Bourque, 2010). We therefore sought to test whether osmotic activation of ΔN-TRPV1 current also depends on regulation of F-actin and whether this process is defective in PLCδ1 KO mice. The top images in Figure 5 are representative images of F-actin labeling (using Texas Red-X Phalloidin) in MNCs isolated from control mice in isotonic solution and following a 15 min exposure to either hypertonic solution or Ang II (0.1 μM). MNCs isolated from control mice showed an increase in F-actin labeling both in response to hypertonic solution and in response to Ang II (which is consistent with what was observed in rat MNCs) (Zhang et al., 2007; Zhang and Bourque, 2008). These data support the hypothesis that increases in osmolality, like treatment with Ang II, may exert some or all its effects on ΔN-TRPV1 channels through regulation of F-actin. Data on the intensity of F-actin labeling in the three conditions are summarized in Figure 5B. The level of staining in isotonic solution (60.2 ± 1.7 arbitrary units; iso: N = 7) is increased significantly by exposure to hypertonic

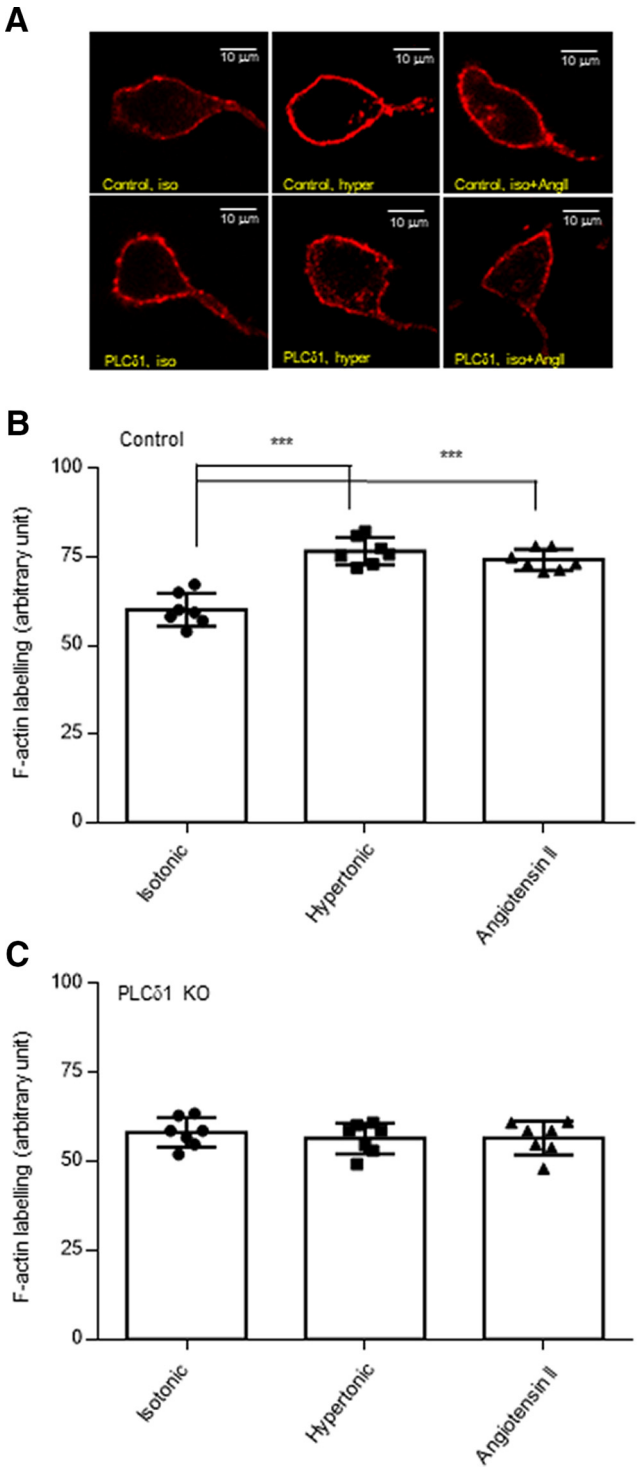

Figure 5. The regulation of F-actin by hypertonic solution and Ang II in MNCs isolated from control and PLCδ1 KO mice. ***A***, Representative images of MNCs showing F-actin fluorescence after the administration of isotonic solution (left), hypertonic solution (middle), and isotonic solution plus Ang II for 15 min in MNCs isolated from control (top images) and PLCδ1 KO mice (bottom images). Scale bar, 10 μm. ***B***, Bar-scatter plots showing F-actin labeling (arbitrary units) in the three conditions in MNCs isolated from control mice. The amplitude of F-actin labeling in isotonic solution (iso) was 60.2 ± 1.7 ($N = 7$) and was significantly higher in the presence of hypertonic solution (hyper; 76.7 ± 3.8, $N = 7$, $p < 0.001$) and Ang II (74.3 ± 2.9, $N = 7$, Bonferroni's *post hoc* test, $p < 0.001$). ***C***, Bar-scatter plots showing F-actin labeling (arbitrary units) in the three conditions in MNCs isolated from PLCδ1 KO mice. The amplitude of F-actin labeling in isotonic solution (iso) was 58.2 ± 4.1 ($N = 7$) and was not significantly higher in the presence of hypertonic solution (hyper; 56.5 ± 4.3, $N = 7$, $p < 0.001$) or of Ang II (56.6 ± 4.7, $N = 7$, Bonferroni's *post hoc* test, $p > 0.05$). The values shown are the mean of the means of each experiment, which included at least 15 cells from each mouse. *N* and *n* indicate the number of animals and cells, respectively. ***$p < 0.001$.

solution (76.7 ± 3.8 arbitrary units; hyper; $N = 7$; $p < 0.001$) and by treatment with Ang II (74.3 ± 2.9 arbitrary units; Ang II; $N = 7$; $p < 0.001$). The bottom images in Figure 5*A* are representative images of F-actin labeling in MNCs isolated from PLCδ1 KO mice in isotonic solution, in hypertonic solution, and in isotonic solution containing Ang II. Both treatments failed to increase F-actin fluorescence (Fig. 5*C*). The level of staining in isotonic solution (58.2 ± 4.1 arbitrary units; iso; $N = 7$) is not changed significantly by exposure to hypertonic solution (56.5 ± 4.3 arbitrary units; hyper; $N = 7$; $p > 0.05$) or by treatment with Ang II (56.6 ± 4.7 arbitrary units; Ang II; $N = 7$; $p > 0.05$). These data suggest that osmotic- and Ang II-dependent regulation of F-actin in MNCs are both defective in PLCδ1 KO mice.

Discussion

We showed previously that PLC is activated by increased osmolality in isolated rat MNCs and that activation of PLC is necessary for full activation of ΔN-TRPV1 currents (Bansal and Fisher, 2017). The potential importance of PLC in MNC osmosensitivity and osmoregulation led us to attempt to identify the isoform of PLC that performs this role. Our observation that the effect is dependent on the concentration of external Ca^{2+} and may be suppressed by antagonists of L-type Ca^{2+} channels (Bansal and Fisher, 2017) led us to hypothesize that the responsible isoform might be Ca^{2+}-dependent and to focus on members of the PLCδ family, which are known to be Ca^{2+}-dependent (Suh et al., 2008). MNCs express PLCδ1 (Hazell et al., 2012), and we therefore sought to determine whether transgenic mice in which this isoform had been knocked out would display abnormal osmoregulation. We subjected a control strain (C57BL/6J) and PLCδ1 KO mice to water deprivation lasting 24 h (which is similar to the protocol that we have used to demonstrate enhanced expression of L-type Ca^{2+} channels in MNCs following water deprivation) (Zhang et al., 2007) and compared their serum osmolality with mice that had free access to water. PLCδ1 KO mice showed a markedly larger increase in osmolality than did control mice, suggesting that PLCδ1 may play a role in osmoregulation (Fig. 1). PLCδ1 KO mice also showed a larger loss of body weight than did control mice following water deprivation. These experiments are consistent with the hypothesis that PLCδ1 mice failed to increase VP release during the osmotic challenge posed by water deprivation and that the mice therefore lost more water through urination than did the control mice. We did not measure VP levels because VP is released by stress and measurements of serum VP levels are therefore confounded by the stress associated with handling and anesthetizing mice (Watts and Swanson, 1987; Russell and Lightman, 2019). The larger increase in plasma osmolality and the larger loss of body weight in PLCδ1 KO mice are consistent with the hypotheses that PLCδ1 is activated by water deprivation in MNCs *in situ*, that PLCδ1 is required for the full activation of ΔN-TRPV1 channels, and that PLCδ1 therefore plays an important role in MNC osmosensitivity.

We therefore sought to determine whether the response of ΔN-TRPV1 channels to osmotic stimulation is defective in MNCs isolated from PLCδ1 KO mice. We furthermore sought to determine whether the osmotic activation of PLC regulates

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the actin cytoskeleton (as has been shown for Ang II-dependent activation of PLC) (Zhang and Bourque, 2008) and whether this process is disrupted in MNCs isolated from PLCδ1 KO mice. We studied the osmotic activation of MNC firing in the control strain and in PLCδ1 KO mice by administering hypertonic saline. As is shown in Figure 2, hypertonic solutions caused a significant increase in the firing of MNCs isolated from the control strain of mice but caused no significant increase in MNCs isolated from the PLCδ1 KO mice. This supports the hypothesis that PLCδ1 plays a role in MNC osmosensitivity. We then demonstrated in voltage-clamped MNCs isolated from control mice that osmotic stimulation activates a nonselective cation current with a reversal potential (−41.6 ± 5.6 mV) that was similar to that seen for the osmotically activated current in rat MNCs (−41 mV) (Oliet and Bourque, 1994) but that the amplitude of this current is dramatically lower in MNCs isolated from PLCδ1 KO mice (Fig. 4). We conclude from these experiments that osmotic activation of ΔN-TRPV1 and MNC firing is defective in PLCδ1 KO mice. Our previous work showed that the osmotic activation of PLC in isolated MNCs is prevented by the presence of the TRPV1 antagonist SB366791 (Bansal and Fisher, 2017). We therefore hypothesize that osmotic stimulation leads to mechanical activation of ΔN-TRPV1 in MNCs in control mice and that the resultant Ca^{2+} influx leads to activation of PLCδ1 and a potentiation of ΔN-TRPV1 activation. We further hypothesize that mechanical activation of ΔN-TRPV1 also occurs in the MNCs of PLCδ1 KO mice and that this is responsible for the small increase in current that we observed (see Figs. 3, 4). The fact that we did not see significant increases in osmotically activated current or cell firing in MNCs isolated from PLCδ1 KO mice might result from limitations in our ability to detect small changes.

Our last set of experiments sought to determine whether the mechanisms by which PLCδ1 regulates ΔN-TRPV1 channels involve regulation of the MNC cytoskeleton. PLC catalyzes the breakdown of plasma membrane phosphatidylinositol 4,5-bisphosphate (PIP_2) into diacylglycerol (which activates PKC) and inositol 1,4,5-trisphosphate (which activates the release of Ca^{2+} from internal stores). Each of these molecules regulates multiple ion channels types. PIP_2 itself regulates >30 types of ion channels, including TRP channels (Suh and Hille, 2005). PLC activation could therefore regulate ΔN-TRPV1 activity through several pathways, including direct effects of PIP_2 (Poblete et al., 2015), activation via PKC (Liu et al., 2020), or Ca^{2+}/calmodulin (Hasan and Zhang, 2018), or translocation of channels to the plasma membrane (Cerny and Huber, 2011; Toro et al., 2011). Any or all these mechanisms could contribute to the osmotic activation of TRPV1 currents in MNCs, and it is possible or even likely that multiple mechanisms are involved with different time courses. We chose to focus on testing whether PLCδ1 regulates the mechanosensitivity of ΔN-TRPV1 channels through an action on the MNC cytoskeleton, as this was shown to be a mechanism by which Ang II exerts its PLC-dependent effects on ΔN-TRPV1 activity (Zhang et al., 2007; Zhang and Bourque, 2008). MNCs display a thin layer of F-actin and a "unique interweaved microtubule scaffold" that is very different from the structure of microtubules in neurons from other brain areas (Prager-Khoutorsky et al., 2014; Barad et al., 2020). The osmotic activation of ΔN-TRPV1 occurs in a cytoskeleton-dependent fashion (Prager-Khoutorsky, 2017), with both cortical actin (Zhang et al., 2007) and microtubules (Prager-Khoutorsky et al., 2014) playing a role. The MNC cytoskeleton and its relationship with ΔN TRPV1 channels are regulated by PLC (Zhang et al., 2007; Prager-Khoutorsky and Bourque, 2010). Neurons in the subfornical organ are excited by systemic hypovolemia and have excitatory inputs onto MNCs that depend on the release of the peptide hormone Ang II (Jhamandas et al., 1989; Li and Ferguson, 1993). Ang II increases the responsiveness of MNCs to changes in external osmolality (Chakfe and Bourque, 2000) by amplifying mechanosensory transduction through a PLC- and PKC-dependent increase in F-actin density (Zhang et al., 2007; Prager-Khoutorsky and Bourque, 2010). These data are consistent with evidence showing that Ang II can cause rapid increases in F-actin density in hippocampal neurons (Pilpel and Segal, 2004) and in other cell types (Aoki et al., 1998; Kuwahara and Kuwahara, 2002).

The fact that Ang II acts on F-actin through a PLC-dependent mechanism led us to speculate that the osmotic activation of PLC in MNCs might also alter F-actin. We demonstrated in MNCs isolated from control mice that osmotic stimulation causes an increase in F-actin density that is like that observed following treatment with Ang II (Fig. 5*A*,*B*). These data suggest that activation of PLC osmotically or through Ang II treatment enhances F-actin through a similar pathway. When we repeated these experiments in MNCs isolated from PLCδ1 KO mice, however, we found that neither osmotic stimulation nor Ang II caused a significant increase in F-actin density. The latter observation is somewhat surprising because Ang II-dependent activation of PLC is thought to depend on the PLCβ isoform (Suh et al., 2008). The Ang II receptor Type 1 (AT_1 receptor) is dominant in MNCs (Culman et al., 1995) and the AT_1 receptor is known to mediate its actions through PLCβ (Poitras et al., 1998). A possible explanation for this observation is presented in the paragraph below.

Our data suggest that the PLCδ1 isoform plays a key role in the full activation of ΔN-TRPV1. This is particularly interesting because, although PLCδ isoforms are known to be involved in a variety of physiological functions (including in sperm, skin, and cardiac myocytes), little is known about the role of these isoforms in neurons (Kadamur and Ross, 2013; Nakamura and Fukami, 2017). One model that has been proposed for the actions of members of the PLCδ family is they might serve to potentiate the responses initiated by the activation of isoforms of other PLC families (Rebecchi and Pentyala, 2000). In this model, activation of other isoforms from other PLC families leads to inositol 1,4,5-trisphosphate-dependent release of intracellular Ca^{2+}, which leads to the activation of PLCδ isoforms and the potentiation of the response. This could explain why the increase in the density of F-actin following either hypertonic saline or treatment with Ang II was prevented by the absence of PLCδ1; it could be that the final effector in both cases is PLCδ1. Osmotic activation could activate PLCδ1 primarily through the activity-dependent influx of Ca^{2+}, whereas in the case of Ang II treatment, it may be that PLCδ1 is activated by Ca^{2+} release from intracellular stores as a result of the Ang II receptor-dependent activation of PLCβ. Our data therefore may have identified an important signaling role for PLCδ1 in neurons that can be initiated by activity-dependent Ca^{2+} influx or through the amplification of signals initiated by other isoforms of PLC. In MNCs, initiation of PLCδ1 through either pathway contributes to a long-lasting change in neuronal excitability through activation of ΔN-TRPV1 channels. PLCδ1 could be involved in similar forms of neuromodulation in other types of neurons.

In conclusion, PLCδ1 plays a central role in osmosensory transduction through the upregulation of F-actin and the activation of ΔN-TRPV1 channels in supraoptic MNCs. The PLCδ1 isoform is activated during MNC action potential firing and exerts positive feedback on ΔN-TRPV1 channels to enhance MNC excitability. PLCδ1 may be an important component of

the mechanisms underlying osmosensory transduction, the regulation of VP release, and mammalian osmoregulation.

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