

# PKC- $\zeta$ Mediates Norepinephrine-Induced Phospholipase D Activation and Cell Proliferation in VSMC

Jean-Hugues Parmentier, Philip Smelcer, Zoran Pavicevic, Edin Basic, Azra Idrizovic, Anne Estes, Kafait U. Malik

**Abstract**—Norepinephrine (NE) stimulates phospholipase D (PLD) activity and cell proliferation in vascular smooth muscle cells (VSMCs). The objective of this study was to determine the contribution of PKC- $\zeta$  to NE-induced PLD activation and cell proliferation in VSMCs. PLD activity was measured by the formation of [ $^3$ H]phosphatidylethanol in VSMCs labeled with [ $^3$ H]oleic acid and exposed to ethanol. A high basal PLD activity was detected, and NE increased PLD activity over basal by 70%. This increase was abolished by the broad-range PKC inhibitor Ro 31-8220 (1  $\mu$ mol/L, 30 minutes) and myristoylated PKC- $\zeta$  pseudosubstrate peptide inhibitor (25  $\mu$ mol/L, 1 hour). Transfection of VSMCs with PKC- $\zeta$  antisense, but not sense, oligonucleotides, which reduced PKC- $\zeta$  protein level and basal PLD activity, caused a 92% decrease in NE-induced PLD activation. NE-induced increase in PLD activity was also reduced by 61% in cells transfected with kinase-deficient FLAG-T410A-PKC- $\zeta$  plasmid but not in those transfected with wild-type PKC- $\zeta$ . NE increased immunoprecipitable PKC- $\zeta$  activity and phosphorylation, reaching a maximum at 2 and 5 minutes, respectively. NE-induced increase in PKC- $\zeta$  activity was inhibited by Ro 31-8220 and by the pseudosubstrate inhibitor. Treatment of VSMCs for 48 hours with PKC- $\zeta$  antisense, but not sense, oligonucleotides also inhibited basal and NE-stimulated cell proliferation by 54% and 57%, respectively, as measured by [ $^3$ H]thymidine incorporation. The inhibitor of PLD activity *n*-butanol, but not its inactive analog *tert*-butanol, also reduced the basal and blocked NE-induced cell proliferation. These data suggest that PKC- $\zeta$  mediates PLD activation and cell proliferation elicited by NE in rabbit VSMCs. (*Hypertension*. 2003;41[part 2]:794-800.)

**Key Words:** phospholipase D ■ norepinephrine ■ muscle, smooth, vascular ■ protein kinases ■ cell proliferation

Norepinephrine (NE), the principal neurotransmitter released from postganglionic sympathetic fibers, maintains vascular tone by causing contraction of vascular smooth muscle cells (VSMCs). It also promotes hypertrophy, hyperplasia, and migration of VSMCs, leading to vascular remodeling, which has been implicated in the development of vascular disease, including hypertension and atherosclerosis.<sup>1-3</sup> NE stimulates VSMC proliferation through  $\alpha_1$ -adrenergic receptors<sup>1</sup> via the activation of the Ras/mitogen-activated protein kinase (MAPK) pathway.<sup>4,5</sup> NE also stimulates phospholipase D (PLD) activity in VSMCs.<sup>6</sup> PLD catalyzes the hydrolysis of phosphatidylcholine into phosphatidic acid and choline. Activation of PLD by neurotransmitters, hormones, or growth factors has been implicated in a wide range of cellular responses, including cellular trafficking, inflammatory and immune response, mitogenesis, cellular differentiation, and apoptosis.<sup>7</sup> To date, 2 PLD isoforms, PLD1 and PLD2, have been cloned and characterized. PLD1 has a low basal activity and is upregulated by small G proteins (ARF, Rho, Ral), protein kinase C (PKC), and phosphatidylinositol 4,5-bisphosphate (PIP<sub>2</sub>) in vitro.<sup>8</sup> In

contrast, PLD2 has a high basal activity, requires PIP<sub>2</sub>, and is upregulated by ARF and PKC.<sup>8</sup> Recently, we demonstrated that NE stimulates PLD activity in rabbit VSMCs via activation of the Ras-extracellular signal-regulated kinase (ERK)1/2 MAPK pathway by a mechanism independent of phorbol ester-dependent PKC.<sup>6</sup> We also reported the selective activation of PLD2 in response to NE and angiotensin II in these cells.<sup>9,10</sup> PLD may contribute to angiotensin II-induced proliferation of VSMCs through the formation of phosphatidic acid, diacylglycerol, and/or its metabolites.<sup>11,12</sup> However, the exact signaling pathway regulating PLD activation in VSMC in response to neurohumoral agents has not yet been established.

The atypical PKC isoforms ( $\iota/\lambda$  and  $\zeta$ ) are both calcium and diacylglycerol independent.<sup>13</sup> PKC- $\zeta$  is a critical mediator of mitogenic signaling in many cell types, mediating angiotensin II-induced activation of ERK and cell proliferation in VSMCs,<sup>14</sup> and insulin-induced ERK activation in adipocytes.<sup>15</sup> The activation of PI 3-kinase by growth factors induces a moderate activation of PKC- $\zeta$  that is mediated by phosphorylation at its T-loop site by 3-phosphoinositide-

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dependent protein kinase-1 (PKD1) followed by a subsequent autophosphorylation.<sup>16–18</sup> PKC- $\zeta$  may also be activated by nonselective binding of acidic lipids such as polyphosphoinositides<sup>18</sup> and phosphatidic acid.<sup>19</sup>

Recent evidence suggests a functional link between atypical isoforms of PKC and PLD activation.<sup>20,21</sup> A novel signaling cascade in Ras-transformed cell lines, in which Ras sequentially stimulates PLD1, PKC- $\iota$ , and PLD2, has been proposed.<sup>22</sup> The present study was conducted to determine the contribution of PKC- $\zeta$  in PLD activation and VSMC proliferation elicited by NE in rabbit VSMCs. To test this hypothesis, we have investigated the effect of interventions that interfere with PKC- $\zeta$  activity on NE-induced PLD activation. We have also examined the consequence of PKC- $\zeta$  and PLD inhibition on VSMC cell proliferation. Our findings suggest that PKC- $\zeta$  acts upstream of PLD, and that PKC- $\zeta$  and PLD regulate both the basal and NE-induced proliferation of VSMCs.

## Methods

### Materials

The following reagents were purchased: [<sup>3</sup>H]oleic acid from American Radiolabeled Chemicals, NE bitartrate from Sigma, phosphatidylethanol and myristoylated PKC- $\zeta$  pseudosubstrate peptide inhibitor from Biomol, Ro 31-8220 from Calbiochem, [<sup>3</sup>H]thymidine and [ $\gamma$ -<sup>32</sup>P]ATP (3000 Ci/mmol) from Amersham, antibody to nPKC- $\zeta$  from Santa-Cruz Biotechnology; phospho-PKC- $\zeta$ / $\lambda$  antibody from Cell Signaling Technology; and anti-FLAG antibody from Sigma.

### Culture of VSMCs

Male New Zealand white rabbits (Myrtle's Rabbitry, Thompson Station, Tenn, maintained according to our institutional guidelines and by the animal care committee at the University of Tennessee, Memphis) were anesthetized with pentobarbital (Abbot Laboratories), and the thorax was opened by an incision. The aorta was rapidly removed, and VSMCs were isolated as previously described.<sup>6</sup> Cells between 3 and 5 passages were plated in 6-well or 24-well plates for experimental assays. Cells were maintained under 5% CO<sub>2</sub> in M-199 medium containing penicillin, streptomycin, amphotericin B, and 10% fetal bovine serum.

### Transient Transfection of VSMCs

The VSMCs were transfected with antisense or sense oligonucleotides designed from the first 7 codons of the rat PKC- $\zeta$  cDNA sequence.<sup>23</sup> Phosphorothioate oligonucleotides (Integrated DNA Technologies) were complexed with Oligofectamine reagent (Invitrogen-Life Technologies) according to the manufacturer's instructions. The oligonucleotide mix was added to the cells for 48 hours before experimental assays. Efficiency of transfection with oligonucleotides was measured by Western blot analysis. For experiments with pCMV5-FLAG-tagged PKC- $\zeta$  plasmids (gift from Dr A. Toker, BBRI, Boston, Mass; and R. Farese, University of South Florida, Tampa, Fla),<sup>13,15</sup> cells in 6-well culture plates were transfected with wild-type PKC- $\zeta$  and kinase-deficient T410A PKC- $\zeta$  for 48 hours using Lipofectamine Plus (Invitrogen-Life Technologies). Transfection efficiencies were determined by Western blot analysis according to the manufacturer's instructions.

### PLD Assay

PLD activity in VSMCs was assayed as described.<sup>10</sup>

### Western Blot Analysis

The efficiency of transient transfection with both oligonucleotides and plasmids was determined by Western blot analysis. Briefly, VSMC treated with oligonucleotides were washed twice and scraped in ice-cold PBS. Cells were pelleted by quick centrifugation and

resuspended in boiling Laemmli sample buffer. Proteins were separated by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. Blocking was performed with TBS buffer (20 mmol/L Tris-HCl at pH 7.6, 200 mmol/L NaCl) containing 5% nonfat dry milk powder. The membrane was then incubated with nPKC- $\zeta$  antibody (1:200) overnight. The membranes were subsequently washed, incubated with HRP-linked secondary antibodies, rinsed, and developed with enhanced chemiluminescence reagents (Amersham). For transfection efficiencies with FLAG-tagged PKC- $\zeta$  plasmids, the top aqueous layer and insoluble fraction from the PLD assay were precipitated with 4 volumes of ice-cold acetone, incubated 1 hour at –80°C, and pelleted and dried under nitrogen. The pellet was resuspended in boiling Laemmli buffer and treated as described above. Ectopic expression of FLAG-PKC- $\zeta$  was detected with an anti-FLAG antibody.

### PKC- $\zeta$ Assay

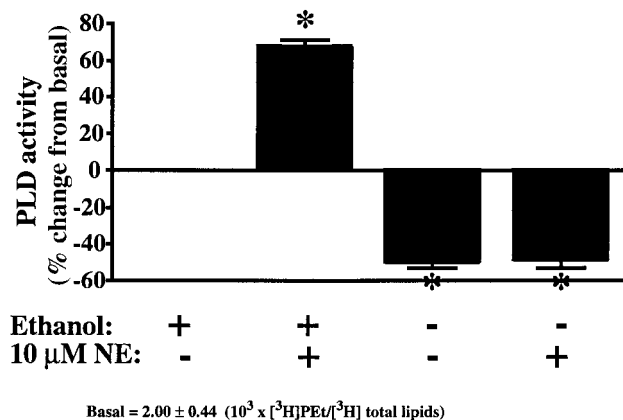
The activity of PKC- $\zeta$  was determined according to the method described.<sup>24</sup> VSMCs in 100-mm dishes were washed twice with PBS and scraped in RIPA buffer. Cells lysates were incubated with rabbit polyclonal PKC- $\zeta$  antibody (Santa Cruz Biotechnologies) for 3 hours, and the immunocomplex was captured with a 50% slurry of protein A agarose beads. PKC- $\zeta$  immunoprecipitates were washed twice with high salt (50 mmol/L Tris.Cl at pH 7.5; 10 mmol/L MgCl<sub>2</sub>, 0.5 mol/L LiCl) and low salt (50 mmol/L Tris.Cl at pH 7.5, 10 mmol/L MgCl<sub>2</sub>) buffers and incubated with a kinase buffer (50 mmol/L Tris.Cl at pH 7.5, 10 mmol/L MgCl<sub>2</sub>, 0.2 mmol/L EGTA, 50  $\mu$ mol/L ATP) containing 50  $\mu$ mol/L  $\epsilon$ -peptide and 3  $\mu$ Ci [ $\gamma$ -<sup>32</sup>P]ATP at 30°C. The reaction was stopped by the addition of 200 mmol/L EDTA, and the proteins were precipitated by the addition of 25% TCA. The solutions were centrifuged for 1 minute at 14 000 rpm, and supernatants were spotted onto p81 phosphocellulose filters. Filters were washed with 1% (v/v) orthophosphoric acid and analyzed by Cerenkov counting. PKC- $\zeta$  activity was calculated from the amount of <sup>32</sup>P incorporated into the  $\epsilon$ -peptide. The phosphorylation of PKC- $\zeta$  on Thr410, required for PKC- $\zeta$  activation by PDK1, was also measured by Western blot analysis using a phospho-PKC- $\zeta$ / $\lambda$  antibody. Membranes were subsequently stripped off in stripping buffer (100 mmol/L  $\beta$ -mercaptoethanol, 62.5 mmol/L Tris · Cl at pH 6.7, 2% SDS) and reprobed with nPKC- $\zeta$  antibody. Densitometric analysis of the bands from the Western blot films was performed with National Institutes of Health Image 1.62 software.

### Cell Proliferation

VSMC proliferation was determined by quantitating the incorporation of [<sup>3</sup>H]thymidine as an indicator of DNA synthesis.<sup>5</sup> Subconfluent cells cultured in 24-well plates were incubated in serum-free M199 for 24 hours to induce mitotic quiescence and transfected with 200 nmol/L PKC- $\zeta$  antisense or sense oligonucleotides, *n*-butanol, or *tert*-butanol for 48 hours. NE or vehicle was added 2 hours after the inhibitors. [<sup>3</sup>H]thymidine (0.25  $\mu$ Ci/well) was added 4 hours before processing the cells. Cells were washed 3 times with PBS, 10% TCA, and ethanol/ether (2:1). <sup>3</sup>H-labeled DNA was extracted with 0.1% SDS/0.1 N NaOH, and radioactivity was measured by scintillation spectroscopy. [<sup>3</sup>H]thymidine incorporation was measured as counts per minute per well and expressed as percentage increase above basal.

### Statistical Analysis

Results are expressed as the mean  $\pm$  SD from different batches of cells for PLD or PKC- $\zeta$  activity. Data were analyzed by 1-way ANOVA. Student *t* test was applied to determine difference between treatments and their respective control values. The Newman-Keuls multiple-range test was applied for comparison of treatments among multiple groups. The null hypothesis was rejected at *P* < 0.05. The phospho-PKC- $\zeta$ /total PKC- $\zeta$  ratio was estimated from the densitometric analysis and arbitrarily chosen as 1.00 for vehicle and expressed as fold increase for the different treatments. Mean  $\pm$  SD of the ratio was calculated from 3 different Western blot analysis.



**Figure 1.** Effect of ethanol removal on NE-induced PLD activity in VSMCs. [ $^3\text{H}$ ]oleic acid-labeled VSMCs were preincubated with or without 200 mmol/L ethanol for 10 minutes and incubated with 10  $\mu\text{mol/L}$  NE or vehicle for 15 minutes. PLD activity was measured as described in Methods. Data are expressed as the percent change in PLD activity over the basal activity in unstimulated cells in presence of ethanol. Values are the mean  $\pm$  SE of 3 independent experiments performed in duplicate. \* $P < 0.05$  vs vehicle with ethanol.

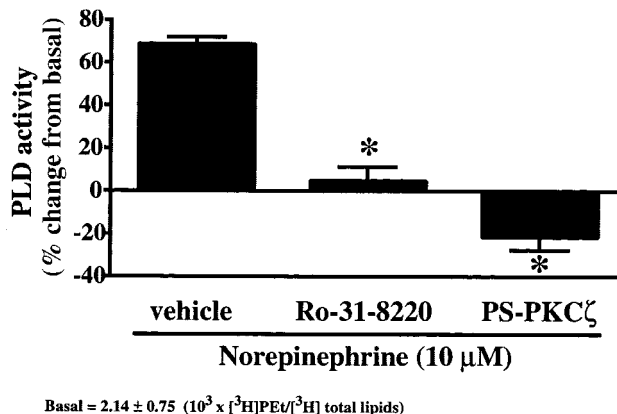
## Results

### Characterization of NE-Induced PLD Activity in Rabbit VSMCs

PLD catalyzes the hydrolysis of phosphatidylcholine into phosphatidic acid and choline. PLD activity was measured by the formation of [ $^3\text{H}$ ]phosphatidylethanol in VSMCs labeled with [ $^3\text{H}$ ]oleic acid and exposed to ethanol. In unstimulated VSMCs, the value of [ $^3\text{H}$ ]phosphatidylethanol/[ $^3\text{H}$ ]total lipids, which was in the range of 2000 to 3000 counts per minute, represented the basal PLD activity. The same procedure performed in the absence of ethanol, which accounts for non-PLD activity,<sup>25</sup> resulted in a 50% lower count than in presence of ethanol (Figure 1). Therefore, PLD is constitutively active in unstimulated rabbit VSMCs, a feature of the PLD2 isoform expressed in these cells.<sup>9,10</sup> However, in other cell types, such as rat-1 fibroblasts, basal PLD activity is similar in the absence or presence of ethanol, suggesting the absence of constitutive PLD activity. NE, at the optimal concentration of 10  $\mu\text{mol/L}$ ,<sup>6</sup> increased PLD activity by 65% to 70% but did not modify basal non-PLD activity in VSMCs (Figure 1).

### PKC- $\zeta$ Mediates NE-Induced PLD Activation

We have previously reported that the Ras/MAPK pathway mediates NE-induced PLD activation in VSMCs.<sup>6</sup> In the same study, we showed that treatment of VSMCs with bisindolylmaleimide I, a PKC inhibitor, or chronic treatment with phorbol 12-myristate 13-acetate that depleted PKC, did not decrease NE-induced PLD activation. These results excluded classical and new PKC isoforms as mediators of PLD activation. Atypical PKCs such as PKC- $\zeta$  or PKC- $\iota/\lambda$  are not sensitive to bisindolylmaleimide I or to phorbol esters. To assess the contribution of atypical PKC to PLD activation, we examined the effects of Ro 31-8220, a broad-range PKC inhibitor, and myristoylated PKC- $\zeta$  pseudosubstrate peptide inhibitor, a selective inhibitor of PKC- $\zeta$ , on NE-induced PLD

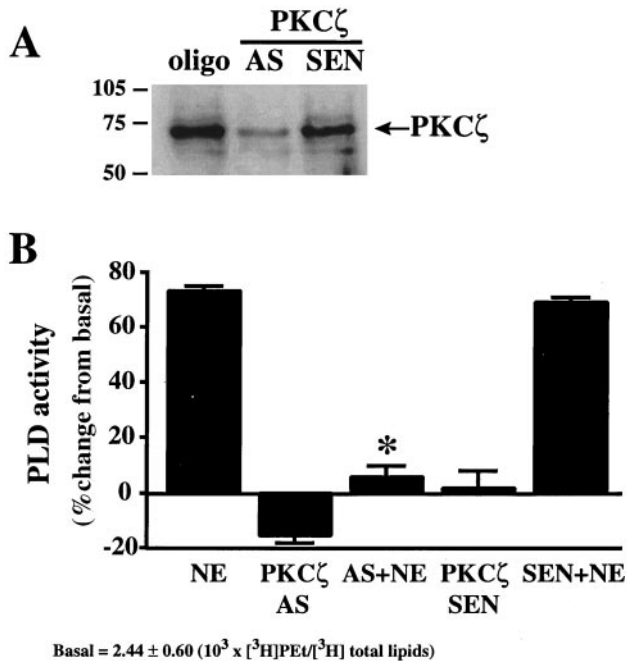


**Figure 2.** Effect of PKC- $\zeta$  inhibition on NE-induced PLD activity. Cells were incubated with 1  $\mu\text{mol/L}$  Ro 31-8220 (30 minutes) (an inhibitor of all PKC isoforms, including PKC- $\zeta$ ) or 25  $\mu\text{mol/L}$  myristoylated PKC- $\zeta$  pseudosubstrate peptide (PS-PKC $\zeta$ , 1 hour; a selective cell-permeable inhibitor of PKC- $\zeta$ ). Cells were then exposed to 10  $\mu\text{mol/L}$  NE. PLD activity was measured as described in Methods. Data are expressed as the percent change in PLD activity from the basal activity. Values are the mean  $\pm$  SE of 4 independent experiments performed in duplicate. \* $P < 0.05$  vs vehicle with ethanol.

activation in VSMCs. Ro 31-8220 (1  $\mu\text{mol/L}$ , 30 minutes) and the PKC- $\zeta$  pseudosubstrate inhibitor (25  $\mu\text{mol/L}$ , 1 hour) decreased NE-induced increase in PLD activity by 93% and by 22% below the basal activity, respectively (Figure 2). To further determine the role of PKC- $\zeta$  in the PLD signaling pathway, VSMCs were transfected with antisense PKC- $\zeta$  oligonucleotide to deplete PKC- $\zeta$  protein levels (Figure 3A). NE-induced PLD activity was inhibited in cells transfected with PKC- $\zeta$  antisense (92% decrease versus NE alone), whereas in cells transfected with PKC- $\zeta$  sense, the PKC- $\zeta$  protein level or PLD activation by NE was not altered (Figure 3B). To further establish the contribution of PKC- $\zeta$  to PLD activation, we examined the effect of NE on PLD activity in VSMCs transiently transfected with wild-type and kinase-deficient T410A PKC- $\zeta$ .<sup>13,15</sup> Figure 4A shows the ectopic expression of FLAG-tagged PKC- $\zeta$  constructs in VSMCs. Basal PLD activity was decreased by 19% in cells transfected with kinase-deficient T410A PKC- $\zeta$  and was unaltered in cells transfected with wild-type PKC- $\zeta$ , (Figure 4B), which is consistent with the result of the oligonucleotides experiment. Overexpression of kinase-deficient T410A PKC- $\zeta$ , but not wild-type PKC- $\zeta$ , markedly reduced NE-induced PLD activation (61% decrease) (Figure 4B). These results suggest that NE activates PLD through PKC- $\zeta$  in rabbit VSMCs.

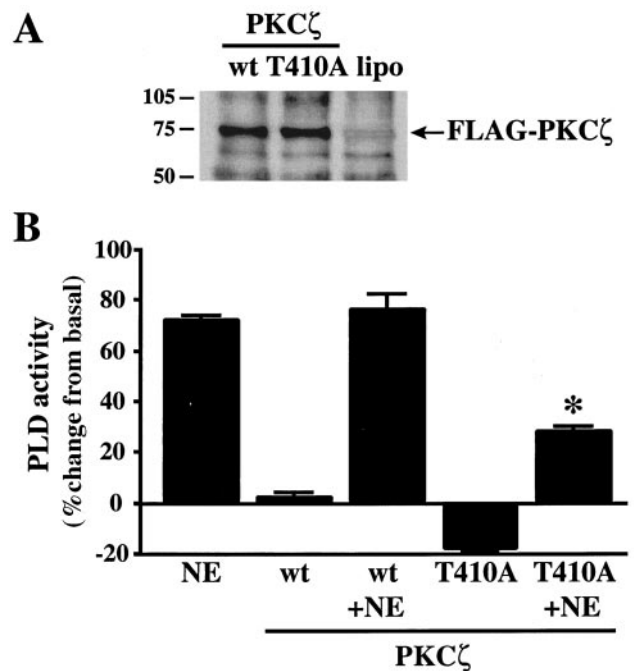
### NE Stimulates PKC- $\zeta$ Phosphorylation and Activity

PKC- $\zeta$  is a 67.7-kDa protein with an apparent molecular weight of 72 kDa on sodium dodecyl sulfate-polyacrylamide gel electrophoresis. In rabbit VSMCs, NE increased the phosphorylation of PKC- $\zeta$  on Thr 410 in a time-dependent manner as shown on Figure 5A (top) using a selective phospho-PKC- $\zeta/\lambda$  antibody. The maximum phosphorylation elicited by NE occurred at 5 minutes. Fetal bovine serum (10%) was used as a positive control for PKC- $\zeta$  phosphorylation. The bottom panel of Figure 5A



**Figure 3.** Effect of PKC- $\zeta$  depletion by antisense oligonucleotides on NE-induced PLD activity. A, Representative Western blot showing the effect of treatment with PKC- $\zeta$  antisense and sense oligonucleotide treatment on PKC- $\zeta$  protein level. VSMCs were incubated with 200 nmol/L PKC- $\zeta$  antisense (AS) or sense (SEN) oligonucleotides, or with vehicle (oligo) for 48 hours, and samples were prepared for Western blot analysis as described in Methods. B, VSMCs were incubated with oligonucleotides as described in A and labeled with [ $^3\text{H}$ ]oleic acid for 18 hours. Cells were exposed to 10  $\mu\text{mol/L}$  NE for 15 minutes. PLD activity was measured as described in Methods. Data are expressed as the percent change in PLD activity from the basal activity in unstimulated oligofectamine-treated cells. Values are the mean  $\pm$  SE of 3 independent experiments performed in duplicate. \* $P < 0.05$ , NE+treatment vs NE alone.

shows the total (phosphorylated and nonphosphorylated) PKC- $\zeta$  protein in VSMCs. The phosphorylated PKC- $\zeta$ /total PKC- $\zeta$  ratio was calculated from the densitometric analysis and shows a time-dependent increase in PKC- $\zeta$  phosphorylation. We also measured the activity of immunoprecipitated PKC- $\zeta$  activity using a selective peptide as a substrate. NE stimulated PKC- $\zeta$  activity in a time-dependent manner, with a peak of 102% increase over vehicle control at 2 minutes of treatment (Figure 5B). Fetal bovine serum (10%) at 1 minute stimulated PKC- $\zeta$  activity to a similar extent as NE at 1 minute. To confirm the selectivity of PKC- $\zeta$  inhibitors that were used to inhibit PLD activity (Figure 2), we measured PKC- $\zeta$  activity in the presence of 1  $\mu\text{mol/L}$  Ro 31-8220 (30 minutes) and 25  $\mu\text{mol/L}$  pseudosubstrate peptide inhibitor (1 hour) after 2 minutes of stimulation with NE. Ro 31-8220 reduced PKC- $\zeta$  activity by 89%, and the pseudosubstrate peptide inhibitor blocked NE-induced increase in PKC- $\zeta$  activity (10  $\mu\text{mol/L}$  NE,  $101.78 \pm 7.02\%$ ; Ro 31-8220+NE,  $10.75 \pm 4.42\%$ ; pseudosubstrate+NE,  $-7.08 \pm 6.91\%$ ; basal,  $7535 \pm 2611$  dpm). Taken together, these results demonstrate the activation of PKC- $\zeta$  by NE in rabbit VSMCs.



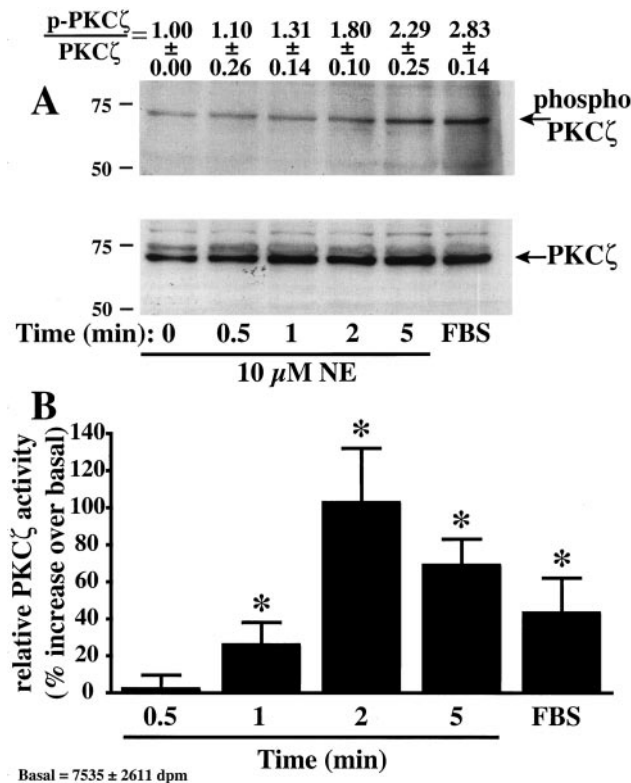
**Figure 4.** Effect of kinase-deficient PKC- $\zeta$  on NE-induced PLD activity in VSMC. A, Representative Western blot showing the expression of FLAG-tagged PKC- $\zeta$  plasmid after transfection. Cells were transfected with wild-type PKC- $\zeta$  and kinase-deficient T410A PKC- $\zeta$ , or lipofectamine alone for 48 hours, and samples were treated for Western blot analysis using a FLAG antibody as described in Methods. B, VSMCs were transfected with plasmids as shown in A and labeled with [ $^3\text{H}$ ]oleic acid for 18 hours. Cells were exposed to 10  $\mu\text{mol/L}$  NE for 15 minutes. PLD activity was measured as described in Methods. Data are expressed as the percent change in PLD activity from the basal activity in unstimulated lipofectamine-treated cells. Values are the mean  $\pm$  SE of 3 independent experiments performed in duplicate. \* $P < 0.05$ , NE+treatment vs NE alone.

### PKC- $\zeta$ and PLD Mediate Cell Proliferation Elicited by NE

VSMC proliferation induced by NE is mediated by the Ras/MAPK pathway in VSMCs.<sup>5</sup> DNA synthesis measured by [ $^3\text{H}$ ]thymidine incorporation was used as a marker for cell proliferation. PKC- $\zeta$  depletion using antisense oligonucleotides inhibited basal [ $^3\text{H}$ ]thymidine incorporation by 54% and NE-induced DNA synthesis by 57%, respectively (Figure 6A). PKC- $\zeta$  sense oligonucleotide did not alter basal- or NE-induced [ $^3\text{H}$ ]thymidine incorporation. *n*-butanol, but not *tert*-butanol (a primary alcohol that can act as an acceptor for the phosphatidyl moiety, thereby generating phosphatidylbutanol instead of phosphatidic acid), is able to inhibit NE-induced PLD activity in VSMCs.<sup>10</sup> *n*-butanol inhibited basal [ $^3\text{H}$ ]thymidine incorporation by 50% and blocked NE-induced DNA synthesis ( $-41.8\%$  below basal) (Figure 6B). *tert*-butanol did not alter basal or NE-induced [ $^3\text{H}$ ]thymidine incorporation.

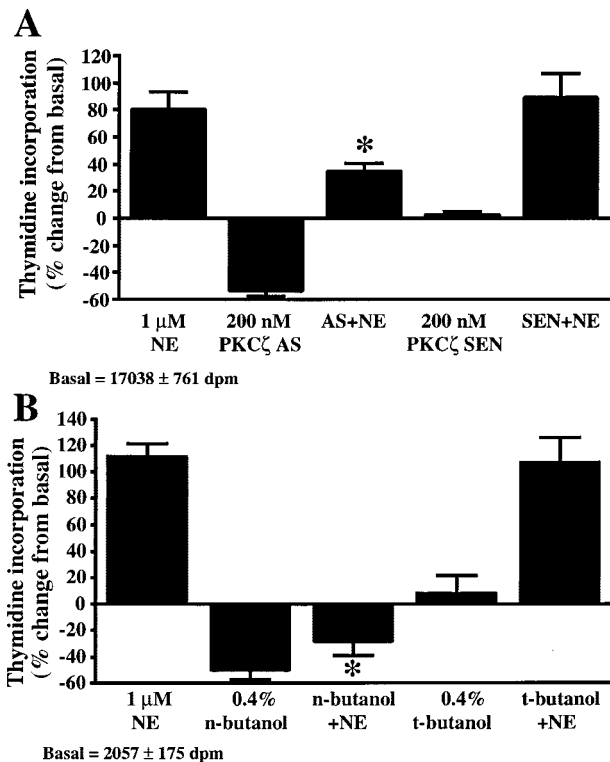
### Discussion

The present study demonstrates the critical role of PKC- $\zeta$  in mediating NE-induced PLD activation and proliferation of



**Figure 5.** Effect of NE treatment on PKC- $\zeta$  activity. A, PKC- $\zeta$  phosphorylation measured by Western blot analysis. Cells were arrested for 48 hours and incubated for different time with 10  $\mu$ mol/L NE. Control was 10% fetal bovine serum (5 minutes). Samples were prepared for Western blot analysis and incubated with a phospho-PKC- $\zeta$ / $\lambda$  (Thr 410/403) antibody (A, top) as described in Methods. The same membranes were stripped off and reprobed with nPKC- $\zeta$  antibody (A, bottom). The phospho-PKC- $\zeta$ /total PKC- $\zeta$  ratio was calculated from densitometric analysis as described in Methods. B, PKC- $\zeta$  activity measured by kinase assay. Cells were arrested and treated with NE as shown in panel A. Cells were lysed and immunoprecipitated with nPKC- $\zeta$  antibody for a kinase assay using [ $\gamma$ - $^{32}$ P]ATP and a selective peptide substrate as described in Methods. Values are the mean  $\pm$  SE of 5 independent experiments and are expressed as the percent increase over basal PKC- $\zeta$  activity. \* $P$  < 0.05 vs basal.

VSMCs. In rabbit VSMCs, NE further activates a constitutively active PLD. Previously, we have shown that NE-induced PLD activation was independent of classical or new types of PKC in VSMCs.<sup>6</sup> However, in the present study, a broad-range PKC inhibitor Ro 31-8220 and the myristoylated PKC- $\zeta$  pseudosubstrate inhibitor abolished NE-induced increase in PLD activity, suggesting the involvement of atypical isoform PKC- $\zeta$  in PLD activation. Supporting this conclusion was our demonstration that the transient transfection of VSMCs with (1) PKC- $\zeta$  antisense, but not sense, oligonucleotide and (2) the dominant-negative PKC- $\zeta$  mutant T410A PKC- $\zeta$ , but not wild-type PKC- $\zeta$ , reduced the basal and blocked the rise in PLD activity elicited by NE. Both PKC- $\zeta$  and PLD have been implicated in the mitogenic action of some growth factors, including angiotensin II.<sup>12,14</sup> Our finding that both PKC- $\zeta$  antisense and inhibition of PLD activity with *n*-butanol reduced basal and NE-induced [ $^3$ H]thymidine incorporation suggests that PKC- $\zeta$ -induced PLD activation is



**Figure 6.** Effect of PKC- $\zeta$  antisense and *n*-butanol on NE-induced PLD activity. VSMCs arrested for 24 hours in 24-well plates were incubated with 200 nmol/L PKC- $\zeta$  antisense (AS), sense (SEN) oligonucleotides, or vehicle (oligofetamine) (A) or with 0.4% *n*-butanol, *tert*-butanol, or vehicle (B) for 48 hours; 1  $\mu$ mol/L NE was added after 2 hours, and 0.25  $\mu$ Ci [ $^3$ H]thymidine was added for the last 4 hours. Samples were prepared as described in Methods. Data are expressed as the percent change in [ $^3$ H]thymidine incorporation from the basal activity. Values are the mean  $\pm$  SE of 8 independent experiments performed in quadruplicate. \* $P$  < 0.05, NE+treatment vs NE alone.

required for the maintenance and induction of cell proliferation elicited by NE in VSMCs.

The mechanism of regulation of PLD activity and cell proliferation by PKC- $\zeta$  in response to NE is not known. The promitogenic signal activated by NE in VSMCs has been shown to involve the Ras/ERK1/2 MAPK pathway.<sup>5</sup> The activation of ERK1/2 by NE appears to be independent of classical or new PKC isoforms, because phorbol 12-myristate 13-acetate (PMA)-induced downregulation of PKCs did not alter NE-induced ERK1/2 MAPK activation.<sup>6</sup> On the other hand, angiotensin II-induced growth/hyperplasia of VSMCs is mediated by EGF receptor transactivation through activation of Ras/MAPK and the PI 3-kinase/PKB/p70 S6 kinase pathway.<sup>26</sup> PKC- $\zeta$  has also been shown to mediate angiotensin II-induced activation of ERK1/2 MAPK in VSMCs.<sup>14</sup> We have previously reported that in VSMC arachidonic acid metabolites, such as 12(S)-, 15(S)-, and 20-HETE, mediate NE- and angiotensin II-induced VSMC proliferation and PLD activation via the Ras/MAPK pathway.<sup>4-6,9,10</sup> Therefore, HETEs may potentially regulate PKC- $\zeta$ -dependent PLD activation and VSMC proliferation elicited by NE and angiotensin II. PKC- $\zeta$  activation by growth factors has also

been reported to be mediated through phosphorylation by PI 3-kinase/PDK1,<sup>17</sup> by binding to acidic lipids (resulting in conformational activation),<sup>18,19</sup> or by interaction with the small G-protein Ras.<sup>14,27</sup> PKC- $\zeta$  also stimulates MAPK/ERK independent of c-Raf.<sup>28</sup> The acidic lipids such as phosphatidic acid, the product resulting from PLD activation, and PIP<sub>3</sub> are able to stimulate PKC- $\zeta$  activity through a phosphorylation-independent mechanism.<sup>18,19</sup> However, in rabbit VSMCs, phosphatidic acid was shown to be required for c-Raf translocation to the plasma membrane but not for its activation.<sup>29</sup> On the other hand, sorbitol and glucose activate PKC- $\zeta/\lambda$  in rat adipocytes, independently of PI 3-kinase, through the ERK pathway and PLD,<sup>20,21</sup> whereas insulin stimulates PKC- $\zeta$  activity through the PIP<sub>3</sub><sup>30</sup> and PDK1-dependent T410 phosphorylation and T560 autophosphorylation of PKC- $\zeta$ .<sup>18,31</sup> In addition, insulin induces Raf-1 translocation and stimulates ERK phosphorylation through PLD2 and phosphatidic acid in rat-1 fibroblasts overexpressing the insulin receptor.<sup>29</sup> Therefore, PLD (ie, phosphatidic acid and ERK) may act upstream of PKC- $\zeta$  in several cell types, including adipocytes and fibroblasts. Therefore, the position of PLD, PKC- $\zeta$ , and ERK in the signaling pathway leading to cell proliferation may differ according to the cell type, (VSMCs versus fibroblasts) and the type of stimulus (NE/insulin versus sorbitol/glucose). In this regard, we have previously reported that ERK1/2 acts upstream in NE- and angiotensin II-induced PLD activation,<sup>6,9</sup> and in the present study, PKC- $\zeta$  was found to mediate PLD activation elicited by NE in VSMCs. In addition, PKC- $\zeta$  mediates angiotensin II-induced ERK activation.<sup>14</sup> Based on these findings, we can postulate that PLD activation by PKC- $\zeta$  is mediated by ERK in response to NE in rabbit VSMCs (PKC- $\zeta$  → ERK → PLD).

Unphosphorylated PKC $\zeta$  is catalytically inactive. Native PKC- $\zeta$  is phosphorylated on Thr 410 in the activation loop of the protein by PDK1. After phosphorylation on Thr 410, PKC- $\zeta$  is autophosphorylated in the activation loop of the catalytic site on Thr 560.<sup>18</sup> These 2 consecutive phosphorylations render PKC- $\zeta$  fully active.<sup>16</sup> In the present study, NE also caused phosphorylation of PKC- $\zeta$  in VSMCs that corresponded with an increase in PKC- $\zeta$  activity, which was attenuated by both Ro 31-8220 and the PKC- $\zeta$  pseudosubstrate peptide inhibitor. However, the maximum phosphorylation seen at 5 minutes with NE stimulation did not correlate with the maximum activity observed at 2 minutes with the kinase assay. Therefore, there is a low correlation between PKC- $\zeta$  phosphorylation at Thr 410 and PKC- $\zeta$  catalytic activity. Catalytic activation of PKC- $\zeta$  may occur before the NE-mediated increase in PKC- $\zeta$  phosphorylation. Our results reflect the possibility that NE stimulates a pool of preactivated PKC- $\zeta$ . Whether other factors, such as interaction with lipids (phosphatidic acid, PIP<sub>3</sub>) or protein/protein interaction, are also involved in the catalytic activation of PKC- $\zeta$  in response to NE is not known. In addition, cellular location and compartmentalization may also affect the selectivity of PKC- $\zeta$  for its targets.<sup>32</sup>

## Perspectives

This study demonstrates that NE-induced PLD activation is mediated by PKC- $\zeta$  in rabbit VSMCs. Furthermore, PKC- $\zeta$

and PLD regulate basal and NE-induced VSMC proliferation. VSMC cell differentiation and/or proliferation are intimately associated with the development of atherosclerosis and malignant hypertension. The nonproliferative phenotype of VSMCs in blood vessels may be modulated toward hyperplasia, hypertrophy, or apoptosis by several pathophysiological stimuli, including NE, through a mechanism involving activation of PLD by PKC- $\zeta$ . Therefore, the development of inhibitors of PKC- $\zeta$  and PLD might prove useful in suppressing smooth muscle cell differentiation and/or proliferation that is associated with atherosclerosis and malignant hypertension.

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## PKC- $\zeta$ Mediates Norepinephrine-Induced Phospholipase D Activation and Cell Proliferation in VSMC

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