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Involvement of Phospholipase D in Norepinephrine Uptake in PC12 Cells

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Phospholipase D (PLD) is an enzyme hydrolyzing phosphatidylcholine to phosphatidic acid (PA) and choline. We investigated the involvement of PLD1 in the uptake of norepinephrine (NE) in PC12 cells, pheochromocytoma cells. NE uptake was specific in PC12 cells because nomifensine, a specific blocker of NE transporter, blocked NE uptake. Inhibition of PLD function in PC12 cells by the treatment of butanol suppressed the NE uptake. In contrast, overexpression of PLD1 in PC12 cells increased NE uptake efficiently. These results suggest that PLD activity is involved in NE uptake. We explored the action mechanism of PLD in NE uptake. PA phosphatase inhibitor, propranolol, blocks the formation of PKC activator diacylglycerol from PA. Propranolol treatment to PC12 cells blocked dramatically the uptake of NE. Specific PKC inhibitors, GF109203X and Ro31-8220, blocked NE uptake. Taken together, we suggest for the first time that PLD1 activity is involved in NE uptake via the activation of PKC.

Key Words: Norepinephrine, Phosphatidic acid, Phospholipase D, Protein kinase C, Transporter, Uptake

INTRODUCTION

Phospholipase hydrolyzes phospholipids, the backbone of biological membranes. Phospholipase D (PLD) hydrolyzes phosphatidylcholine then generates choline and phosphatidic acid (PA), a multifunctional lipid. PA can be further converted to diacylglycerol (DAG), an activator of PKC, by PA phosphatase (Exton, 1997). Hormones, neurotransmitters, cytokines, growth factors (Exton, 1997), and oxidative stress (Oh et al., 2000; Kim et al., 2003) stimulate PLD activity. Studies in HL-60 cells (Stutchfield and Cockcroft, 1993) and platelets (Haslam and Coorsen, 1993) suggested that PLD might participate in regulated exocytosis in an ARF-dependent manner (Fensome et al., 1996; Jones et al., 1999). In neutrophils and RBL-2H3 basophilic leukemia cells, most PLD activity co-localizes with secretory vesicles and translocates to the plasma membrane on stimulation (Morgan et al., 1997; Brown et al., 1998; Choi et al., 2002). In chromaffin cells, secretagogues stimulate the rapid translocation of ARF6 from secretory granules to the plasma membrane and the concomitant activation of PLD in the plasma membrane (Galas et al., 1997; Caumont et al., 1998). By generating fusogenic lipid PA at the exocytic sites, PLD may represent an essential component of the fusion machinery in neuroendocrine cells (Vitale et al., 2001; Humeau et al., 2001).

The catecholamines dopamine, norepinephrine, and epinephrine function both as neurotransmitters and hormones in dopaminergic and adrenergic systems. Specific transport protein, such as a norepinephrine transporter (NET), regulates catecholamine signaling and serves as the main clearance mechanism of secreted ligands in central and peripheral nervous systems (Axelrod and Kopin, 1969). NET malfunction underlies certain patho-physiological conditions, including diabetic cardiomyopathy, congestive heart failure, ischemia-induced arrhythmia and orthostatic intolerance (Ganguly et al., 1986; Meredith et al., 1993; Seyfarth et al.,

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1996; Shannon et al., 2000). Insulin, angiotensin II, atrial natriuretic peptide and nitric oxide also regulate NET functions (Figlewicz et al., 1993; Kaye et al., 1997; Sumners and Raizada, 1986; Vatta et al., 1993), implying an intricate connection between this transporter and other cellular complexes. Human placenta expresses both NET and serotonin transporter (Jayanthi et al., 1993; Ramamoorthy et al., 1993 and 1995). Ramamoorthy et al. showed that NE uptake in brush border membrane vesicles prepared from human term placenta is more sensitive to the NET-specific inhibitor. NE transport plays numerous roles in placental physiology and antidepressants. Drug abuses such as cocaine and amphetamines that block placental NET may adversely affect pregnancy and fetal development (Ramamoorthy et al., 1993 and 1995). PC12 cells originating from rat pheochromocytoma have been known to uptake NE and predominately express the NE transporter (Pacholczyk et al., 1991). Calcium ion-dependent enhancement of the NE uptake in PC12 cells is mediated by activation of calmodulin-dependent protein kinase (Uchida et al., 1998). The small G protein Rab3B stimulates the NE uptake by interacting directly with phosphoinositide 3-kinase (PI3K) and then elevating PI3K activity in PC12 cells (Francis et al., 2002). NE uptake by acute insulin treatment can be stimulated by an enhancement of NET capacity (Vmax) without an alteration in NE Km, whereas the p38 MAPK-dependent enhancement of NET occurs without a detectable enhancement of surface NET (Apparsundaram et al., 2001). PKC down-regulates cell surface NET protein levels and diminish NE uptake capacity (Uchida et al., 1998). Constitutive dopamine transporter trafficking and PKC-mediated its sequestration is achieved by a combination of accelerated internalization and reduced recycling (Loder and Melikian, 2003). The resting tone of NE clearance is established through the modulation of NET surface trafficking and the intrinsic activation of surface-resident NET proteins.

Although the involvements of PLD in the secretion of neurotransmitters and hormones have been studied, the role of PLD in the NE uptake has not been investigated. Thus, to elucidate possible PLD dependence of the NE uptake, we investigated the effects of PLD on NE uptake into PC12 cells. We suggest for the first time that PLD is involved in the uptake of NE through the activation of PKC in PC12 cells.

MATERIALS AND METHODS

Materials

[^{3}H]norepinephrine ([^{3}H]NE) and [9,10-^{3}H] myristic acid (10 Ci/mmol) were purchased from DuPont NEN. PC12 cells were purchased from ATCC (catalog number ATCC CRL 1721). Fetal bovine serum (FBS), RPMI1640 with L-glutamine, penicillin, streptomycin, and G418 were purchased from Life Technologies. Nomifensine, reserpine, propranolol, and 4-phorbol 12-myristate 13-acetate (PMA) were from Sigma. Anti-PLD antibody was a generous gift from Dr. Ryu, S.H. (Pohang University, Korea). GF109203X and Ro31-8220 were from CalBiochem. Nitrocellulose filter and ECL detection kit were from Amersham. Reagents for protein assay were from BioRad. Silica gel 60 Å plate was from Whatman. Phosphatidylbutanol (PtdBut) was a generous gift from Dr. Choi, M.U. (Seoul National University, Korea).

Cell culture

PC12 cells were maintained in a RPMI 1640 medium supplemented with horse serum (10%), fetal bovine serum (5%), penicillin G (100 U/ml) and streptomycin (100 U/ml). The cells were cultured in a humidified environment of 95% air and 5% CO_2 at 37℃. A culture medium and supplements were obtained from Life Technologies. Cells were grown in a culture dish (100 mm; Falcon) and were fed three times per week.

Uptake assays

NE uptake measurements were performed, as described previously (Melikian et al., 1994), by incubating 2×10^5 PC12 cells in each well of 24 well plate for 24 h at 37℃. After removal of culture medium, the cells were incubated 1 h in 500 μl RPMI-1640 medium with [^{3}H]-NE (0.5 μCi/ml) containing ascorbic acid (100 μM). At the same time, propranolol, GF109203X, or reserpine were added to final concentrations 100 μM, 10 μM, or 10 μM, respectively.

Assays were terminated by removing the radiolabel and by rapid washings of cells with 0.5 ml RPMI-1640 medium. Cells were solubilized with 0.3 ml of 0.25 M NaOH. Accumulated radioactivity in cells was quantified by LSC. The histograms are mean with standard error bars for two independent experiments.

PLD assay

PLD activity was determined by measuring the formation of phosphatidylbutanol (PtdBut), a reaction product of PLD in the presence of 0.3% butanol (Oh et al., 2000). PC12 cells (1×10^6 cells per 35 mm plate) were cultured in RPMI-1640 overnight at 37℃ in humidified 5% CO_2. Cells were labeled in RPMI-1640 medium with [^{3}H] myristic acid (1 μCi/ml) for 3 h. Unincorporated [^{3}H] myristic acid was removed by washing with PBS. Butanol and/or GF109203X were then added to a final concentration of 0.3% (vol/vol) and/or 10 μM, respectively. Cells were then incubated for 1 h. The reaction was terminated by the removal of medium and addition of methanol. After extraction of total phospholipids in cells and separation of phospholipids by TLC, the amount of formed PtdBut and total phospholipids were measured with LSC. The data were presented as a percentage of PtdBut per total count. The histograms are mean with standard error bars for two independent experiments.

Immunoblot analysis

Cells were lysed with 20 mM Hepes (pH 7.2) containing 1% Triton X-100, 1% sodium deoxycholate, 10% glycerol, 150 mM NaCl, 50 mM NaF, 1 mM Na_3VO_4, 10 μg/ml leupeptin, 10 μg/ml aprotinin, and 1 mM PMSF. Cell lysates were boiled for 5 min in SDS sample buffer and subjected to 10% SDS-polyacrylamide gel electrophoresis. Proteins were transferred to a nitrocellulose membrane, and blots were incubated for 30 min with 20 mM Tris (pH 7.6), 150 mM NaCl, and 0.1% (v/v) Tween-20 containing 5% (w/v) nonfat dried milk. The membranes were incubated with antibodies recognizing PLD. Blots were washed in 20 mM Tris (pH 7.6), 150 mM NaCl, and 0.1% (v/v) Tween-20. Anti-rabbit IgG antibody coupled with horseradish peroxidase was used for detection of corresponding proteins using ECL reagent.

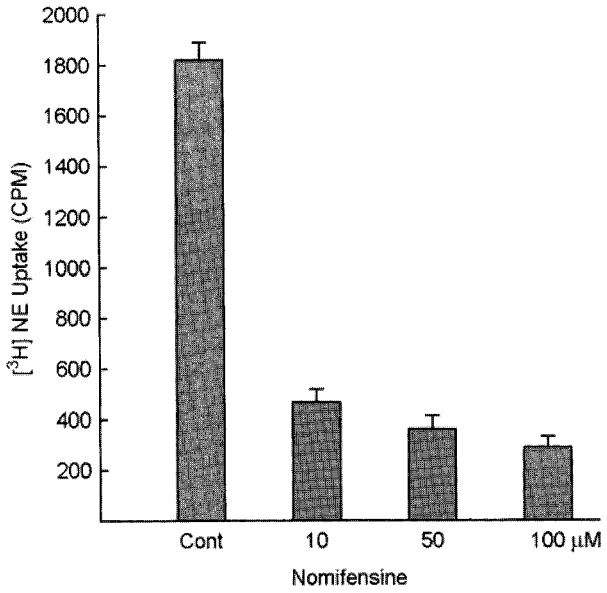

Fig. 1. NE uptake was inhibited by a norepinephrine transporter (NET) blocker nomifensine in a dose-dependent manner in PC12 cells. The cells were incubated 1 h with 500 μl RPMI-1640 medium with [^{3}H]-NE (0.5 μCi/ml) containing ascorbic acid. At the same time, nomifensine of indicated concentrations were added.

RESULTS

Involvement of PLD in NE uptake

We explored the involvement of PLD1 in NE uptake in PC12 cells. We examined the NE uptake is a specific process in PC12 cells. Nomifensine, a specific NET blocker, inhibited NE uptake specifically in the dose dependent manner, a specific NET blocker (Fig. 1). Therefore, we concluded that NE uptake in PC12 cells occurred specifically through a NET.

In the presence of primary alcohol such as butanol, PLD induces transphosphatidylation reaction instead of hydrolysis reaction while PLD cannot do such reaction in the presence of tertiary butanol. Transphosphatidylation reaction makes phosphatidylbutanol instead of PA. Phosphatidylbutanol cannot be metabolized to DAG, a PKC activator, by PA phosphatase. Therefore, the presence of butanol in PLD reaction prevents the normal signaling processes of PLD. We took this advantage to examine the involvement of PLD in NE uptake. Butanol inhibited NE uptake specifically in does dependent manner (Fig. 2). However, 0.6% tertiary

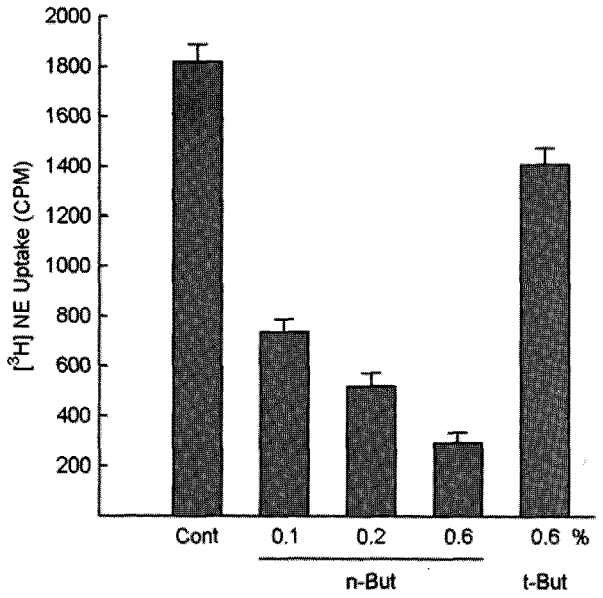

Fig. 2. NE uptake was inhibited by a PLD inhibitor n-butanol in a dose-dependent manner in PC12 cells. The cells were incubated 1 h with 500 μl RPMI-1640 medium with [^{3}H]-NE (0.5 μCi/ml) containing ascorbic acid. At the same time, n-butanol (n-But) or t-butanol (t-But) of indicated concentrations were added.

butanol suppressed NE uptake marginally while 0.1% primary butanol did dramatically. These PLD inhibition studies suggest that the production of PA or DAG from PLD enzyme reaction takes part in NE uptake process.

In contrast to PLD inhibition effect on NE uptake, we explored the overexpression effect on NE uptake. We made PC12-PLD1 cell line, a stable cell line overexpressing PLD1 in PC12 cells (see material and methods of reference 22). Growth and morphology of PC12-PLD1 were similar with control PC12 cells. Western blot data with anti-PLD antibody revealed that PC12-PLD1 cells expressed recombinant PLD1 proteins (Fig. 3A). Enzyme activity of PLD of PC12-PLD1 cells was 7 times higher than control cells (Fig. 3B).

Next, we examined the kinetic of NE uptake of PC12 cells. The cells uptaked [^{3}H]NE linearly to 1 hour. There was uptake saturation at 2 hour (Fig. 4). However, the rate and saturation level of NE uptake in PC12-PLD1 cells were significantly increased compared to control cells (Fig. 4). These experiments strongly suggest PLD1 is positively regulating NE uptake in PC12 cells.

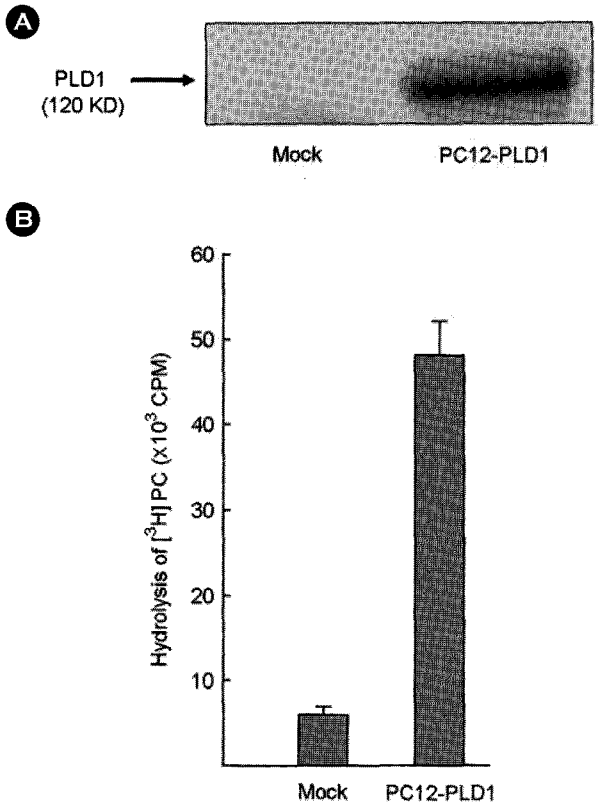

Fig. 3. Overexpression of PLD1 in PC12 cells. PC12 cells were infected with recombinant retrovirus encoding human PLD1. **A:** Western blot analysis of PLD1 in mock and PC12-PLD1 cells. **B:** *In vitro* activity of PLD is increased in PC12-PLD1 cells compared to the mock cells. Cells were lysed by passage through a 29-gauge needle, and lysates (10 μg of protein) were used for the PC head group release assay as described in Materials and Methods.

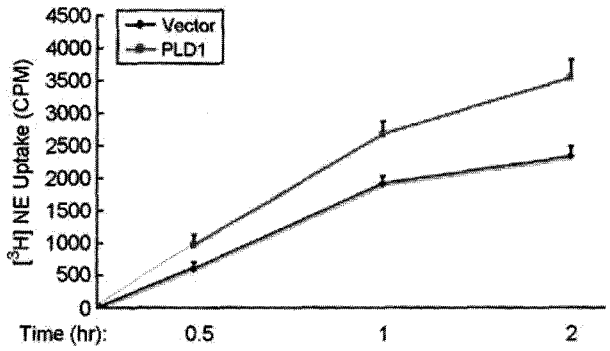

Fig. 4. NE uptake was increased in PC12-PLD1 cells. The cells were incubated 1 h with 500 μl RPMI-1640 medium with [^{3}H]-NE (0.5 μCi/ml) containing ascorbic acid for the indicated times.

Regulation mechanism of NE uptake by PLD1

To investigate the regulation mechanism of the stimulating effect of PLD1 on NE uptake, we employed propranolol,

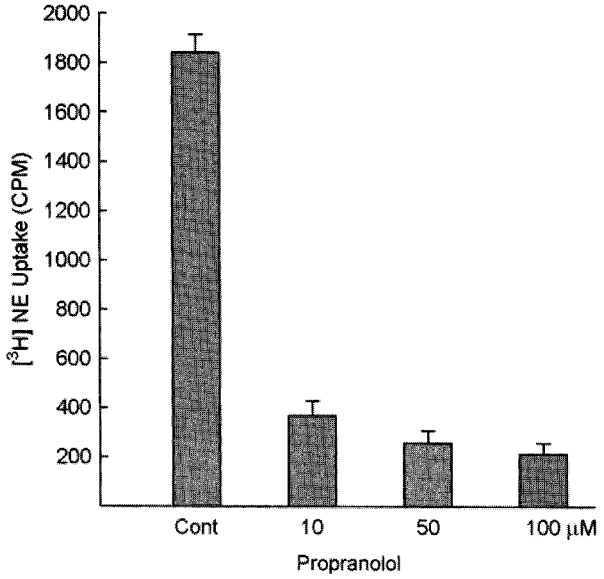

Fig. 5. NE uptake was inhibited by a phosphatidic acid phosphatase inhibitor propranolol in a dose-dependent manner in PC12 cells. The cells were incubated 1 h with 500 μl RPMI-1640 medium with [^{3}H]-NE (0.5 μCi/ml) containing ascorbic acid. At the same time, propranolol was added at the indicated concentration.

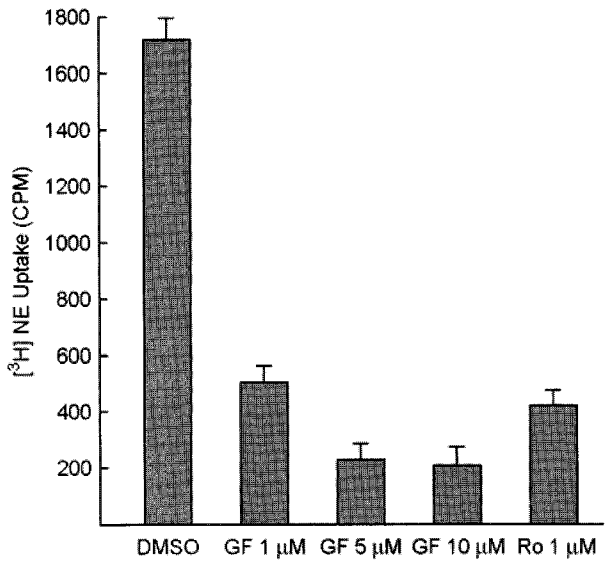

Fig. 6. NE uptake was inhibited by PKC inhibitors, GF109203X and Ro31-8220 in PC12 cells. The cells were incubated 1 h with 500 μl RPMI-1640 medium with [^{3}H]-NE (0.5 μCi/ml) containing ascorbic acid. At the same time, GF109203X (GF) and Ro31-8220 (RO) of indicated concentrations were added.

PA phosphatase inhibitor. PLD hydrolyses phosphatidylcholine to yield PA and choline. Produced PA further metabolized to DAG by PA phosphatase. Propranolol inhibits PA phosphatase to accumulate PA and reduce the production of DAG, an activator of PKC (Exton, 1997). As shown in Fig. 5, the treatment of propranolol to PC12 cells inhibited NE uptake specifically. These results suggest that downstream metabolites of PA maybe involved in PLD1-mediated NE uptake in PC12 cells.

Because formation of DAG from PA, a product of PLD1 activity, activates novel PKCs (Ha and Exton, 1993), we assume that PKC activity is involved in NE uptake. To investigate the involvement of PKC in NE uptake, we treated general PKC inhibitors, GF109203X or Ro31-8220 to PC12 cells. As shown in Fig. 6, GF109203X and Ro31-8220 suppressed NE uptake of PC12 cells dramatically. However, a PKCα isotype specific inhibitor, Go6976, and a PKCδ specific inhibitor, Rottlerin did not show any effect on the NE uptake (data now shown). These results suggest that PKC isozymes are involved in the NE uptake in PC12 cells but PKCα and PKCδ are not.

DISCUSSION

PLD plays major roles at many different steps in vesicle trafficking, including activation of signaling networks, budding of vesicles from the trans-Golgi and vesicle fusion (Humeau et al., 2001). Although its roles in secretion have been extensively studied, the roles in uptake are poorly characterized. In the present study, we showed for the first time that the PLD1 activity is necessary for the NE uptake in PC12 cells.

The first evidence is that NE uptake was inhibited by blockers of PLD signaling pathway including butanol and propranolol. The second evidence is that NE uptake was increased in PC12 cells overexpressing PLD1. These evidences suggest that PLD1 is involved in NE uptake in PC12 cells.

Next question is how PLD1 activity can regulate NE uptake. The inhibition of PLD-dependent NE uptake by propranolol suggests the involvement of PKC in the PLD-dependent NE uptake. There are several isotypes of PKC which have diverse cellular effects. So, different isotypes may have different effects on NE uptakes. Although Go6976

inhibits specifically to PKC-alpha, a classical PKC isotype, it did not suppressed NE uptake in PC12 cells (data not shown). Also, in this experimental condition, there is no calcium ion influx that is necessary for classical PKC activation. These data suggest that the PKC isotype involved in the NE uptake is not a classical PKC isotype. We can assume that other PKC isotype such as a novel PKC isotype is involved in the NE uptake. However, a specific inhibitor to PKCδ Rottlerin showed no effect on NE uptake (data not shown). Therefore, we suggest that a PKC isotype involved in PLD1-mediated NE uptake is a noble PKC isotype excluding PKCδ.

Acknowledgements

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