Dexamethasone enhances phospholipase D activity in M-1 cells

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Abbreviations: ARF, ADP-ribosylation factor; GTP γ S, guanosine 5'-O-(3-thiotriphosphate); PA, phosphatidic acid; PC, phosphatidylcholine; PEth, phosphatidylethanol; PIP₂, phosphatidylinositol 4,5-bisphosphate; PKC, protein kinase C; PLD, phospholipase D; PMA, phorbol 12-myristate 13-acetate

Abstract

Phospholipase D (PLD) is an enzyme involved in signal transduction and widely distributed in mammalian cells. The signal transduction pathways and role for phospholipid metabolism during hormonal response in cortical collecting duct remain partly undefined. It has been reported that dexamethasone increases transepithelial transport in M-1 cells that are derived from the mouse cortical collecting duct. We investigated the expression and activity of PLD in M-1 cells. Basal PLD activity of M-1 cells cultured in the presence of dexamethasone (5 μ M) was higher than in the absence of dexamethasone. Dexamethasone and ATP activated PLD in M-1 cells but phorbol ester did not stimulate PLD activity. Vasopressin, bradykinin, dibutyryl cyclic AMP, and ionomycin were ineffective in activating PLD of the cells. The PLD2 isotype was detected by immunoprecipitation but PLD1 was not detected in M-1 cells. Addition of GTP γ S and ADP-ribosylation factor or phosphatidylinositol 4,5-bisphosphate to digitonin-permeabilized cells did not augment PLD activity. In intact cells PLD activity was increased by sodium oleate but there was no significant change between dexamethasone treated- and untreated cells by oleate. These results suggest that at least two types of PLD

are present in M-1 cells and PLD plays a role in the corticosteroid-mediated response of cortical collecting duct cells.

Keywords: Phospholipase D, dexamethasone, M-1 cell, oleate

Introduction

Mammalian phospholipase D (PLD) activity is very low and becomes up-regulated transiently when cells are stimulated by a variety of hormones, growth factors, and other extracellular signals (Exton, 1997). PLD hydrolyses phosphatidylcholine (PC), the major component of membrane phospholipid to phosphatidic acid (PA) and choline (Bocckino *et al.*, 1987). PA is used as a messenger for signal transduction and can be further metabolized by PA phosphohydrolase to form diacylglycerol and by phospholipase A₂ to form lysophosphatidic acid, which have important physiological functions (Cross *et al.*, 1996; Ktistakis *et al.*, 1996; Jones *et al.*, 1999).

Two PLDs (PLD1 and PLD2) have been cloned and characterized (Hammond *et al.*, 1995; Colley *et al.*, 1997b). PLD1 and PLD2 differ with respect to both regulatory and subcellular localization (Colley *et al.*, 1997b). PLD1 localizes to the perinuclear region (endoplasmic reticulum, Golgi apparatus, and late endosomes) but PLD2 localizes primarily to the plasma membrane. PLD1 has a low basal activity that is increased by regulators including protein kinase C (PKC), tyrosine kinase, Ca²⁺, and low molecular weight GTP-binding proteins such as ADP-ribosylation factor (ARF) and RhoA (Hammond *et al.*, 1997). In contrast, PLD2 exhibits a high basal activity that can be further increased by addition of phosphatidylinositol 4,5-bisphosphate (PIP₂) and oleate but not further activated by PKC, ARF, or Rho *in vitro* (Lopez *et al.*, 1998; Exton, 1999; Kim *et al.*, 1999a). In addition, several groups reported another form of PLD that is activated by detergent including oleate (Chalifour and Kanfer, 1982; Massenburg *et al.*, 1994; Okamura and Yamashita, 1994). Oleate-dependent PLD has been purified from pig lung membranes but it requires further characterization (Okamura and Yamashita, 1994).

M-1 cells have been developed from microdissected cortical collecting duct of a mouse transgenic for the early region of simian virus 40 (Stoos *et al.*, 1991). M-1 cells, which preserve functional properties typical for cortical collecting duct principal cells *in vivo*, reabsorb sodium and secrete potassium through a corticosteroid

regulated Na⁺ channel (Chalfant *et al.*, 1996). Recent studies have demonstrated that dexamethasone stimulates Na⁺ transport in M-1 cells (Nakhoul *et al.*, 1998). Hormones are able to regulate a variety of ion channels indirectly by cytoplasmic pathways involving second messengers (Breyer and Ando, 1994).

It has been reported that both PLD1 and PLD2 are present in mouse kidney but only PLD2 can be detected in human kidney tissue (Colley *et al.*, 1997a; Meier *et al.*, 1999). Activation of PLD and subsequent production of PA are known to be key early event and regulatory in intracellular vesicle trafficking and exocytosis (Jones *et al.*, 1999; Roth, 1999). In epithelial cells, rapid changes in ion transport involve exocytosis and fusion of intracellular vesicles containing transport protein and ion channels with the plasma membrane (Denker and Nigam, 1998). Vesicle trafficking also plays an important role on water reabsorption and secretion of acid or base in cortical collecting duct (Brown, 1989).

However, it is not known whether these hormonal effects involve metabolism and signaling machinery of phospholipid by PLD. The purpose of the present investigation was to determine PLD activity and the effect of agents modulating ion transport on the activity in M-1 cells.

Materials and Methods

Materials

[9,10-³H(N)]myristic acid (10-60 Ci/mmol) was purchased from Dupont-New England Nuclear (Boston, USA). Dexamethasone, phorbol 12-myristate 13-acetate (PMA), guanosine 5'-gamma-thio-triphosphate (GTP γ S), vasopressin, ATP, dibutyl cyclic AMP (dbcAMP), ionomycin, digitonin, protein A-agarose, culture media and sodium oleate were from Sigma. Silica gel 60 TLC plate was purchased from Merck. Authentic phosphatidylethanol (PEth) was from Avanti polar-Lipids Inc. (Alabaster, USA). Recombinant ARF1 was prepared from *Escherichia coli* expressing human ARF1 and yeast myristoyl-CoA: protein *N*-myristoyltransferase (Lambeth *et al.*, 1995). Anti-PLD1 antibody was a generous gift from Dr. Sung Ho Ryu at Pohang University of Science and Technology (Lee *et al.*, 1997). Polyclonal rabbit anti-sera against PLD2 were produced by immunization with a synthetic peptide encoding the 13 amino acids of mouse PLD2 (DRPFEDFIDRETT). The anti-sera recognized mouse PLD1 and PLD2, respectively.

Cell culture

M-1 cells were grown in culture dishes in DMEM/Ham's F-12 (1 : 1 mixture) containing 10% fetal bovine serum, 100 U/ml penicillin, 100 μ g/ml streptomycin. In appropriate experiments, dexamethasone was added to a final concentration of 5 μ M.

Measurement of PLD activity

Assay of PLD activity was based upon the unique ability of this enzyme to stimulate transphosphatidylation in the presence of ethanol to form the corresponding PEth (Kang *et al.*, 1998; Park *et al.*, 1999). M-1 cells were labeled with [³H]myristic acid (1 μ Ci/ml) for 16 h. The cells were washed twice by phosphate buffered saline (PBS) and resuspended in assay buffer (20 mM Hepes, pH 7.4, 137 mM NaCl, 2.7 mM KCl, 3 mM MgCl₂, 2 mM CaCl₂, 2 mM EGTA, and 1 mg/ml bovine serum albumin). After incubation for 20 min at 37°C in the presence of 1.6% ethanol, reactions were stopped by addition of 1 ml of CHCl₃/CH₃OH/concentrated HCl (50 : 50 : 0.3, volume/volume), and 0.35 ml of 1 M HCl/5 mM EGTA. Lipids were extracted and separated on Silica gel 60 TLC plates in a solvent system consisting of ethyl acetate/trimethyl pentane/acetic acid/H₂O (13 : 2 : 3 : 10, volume/volume). The plates were exposed to iodine vapor and [³H]PEth was identified by comigration with PEth carrier. Radioactive [³H]PEth was scraped off the plates and quantitated in a liquid scintillation counter (Beckman LS 5801). The amount of radioactivity in PEth was expressed as percentage of total counts in each lane.

Immunoprecipitation and Western blot of PLD proteins

The cells were lysed in buffer containing 20 mM Tris-HCl, pH 7.4, 50 mM NaCl, 1% Triton X-100, 1% deoxycholic acid, 1 μ g leupeptin, 1 μ g pepstatin A, 1 μ g aprotinin, and 1 mM PMSF. The lysates (10 mg) were incubated with 2 μ g anti-PLD antibody bound to protein A agarose for 1 h (Lee *et al.*, 1997). The beads were washed with PBS containing 1% Triton X-100 three times and further washed with the PBS three times. Cell lysates (100 μ g) and immunoprecipitated beads were subjected to 7.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and then transferred to nitrocellulose membranes. The membranes were blocked for 1 h at 25°C with blocking buffer (10 mM Tris HCl, 0.15 M NaCl, 0.1% sodium azide and 5% skim milk) and incubated with a mixture of primary polyclonal antibodies directed against PLD1 and PLD2 (1 : 1000) in blocking buffer overnight at 4°C. Secondary antibody directed against rabbit IgG conjugated to HRP was diluted 1 : 10,000 in blocking buffer and incubated for 1 h. The signal was detected by enhanced chemiluminescence on Hyperfilm-ECL from Amersham International.

Statistical analysis

Results were expressed as mean values $\pm$ standard deviation of the mean (SD). Student's *t*-test was used to compare the mean PLD activity. A *P* value < 0.05 was considered significant.

Results

PLD activity was measured in the presence of ethanol, as PLD catalyzes the transphosphatidyl reaction that yields the stable product PEth. Cells were cultured in the presence or absence of 5 μ M dexamethasone for 4 days. The level of [3 H]PEth was measured 15 min after incubation of the cells with agonist since no further increase of activity was observed even in the prolonged incubations. As shown in Figure 1, mean basal PLD activity was higher in cells that were cultured in the presence of dexamethasone than in untreated cells ($1.53 \pm 0.05\%$ vs $0.74 \pm 0.1\%$). Phorbol 12-myristate 13-acetate (PMA) treatment has been previously shown to activate PLD activity in various cell systems (Exton, 1997). However, no stimulation was observed with 100 nM PMA for up to 1 h in M-1 cells obtained from both culture conditions (Figure 1). This result shows that activation of PKC is not essential for activation of PLD in M-1 cells. At the same time we found that addition of dexamethasone to cells in culture medium increased transepithelial resistance of M-1 cells (data not shown). We investigated whether dexamethasone directly stimulates the PLD activity of M-1 cells. PLD activity was increased up to 230% by dexamethasone in control M-1 cells (Figure 2). Dose-dependent effect of dexamethasone on PLD activity in both cells was evaluated. Total [3 H]PEth formation in control and dexamethasone-treated cells was obtained by incubation of the cells with 0.1 μ M dexamethasone (Figure 3). When cultured cells in the presence of dexamethasone were treated with same agent, PLD

Figure 1. PLD activity of M-1 cells. M-1 cells which had been cultured in the absence (control) or presence of 5 μ M dexamethasone were radiolabeled with [3 H]myristic acid. The cells at 1×10^6 /ml in buffer containing 1.6% ethanol were incubated with or without 100 nM PMA for 15 min. PEth formation was expressed as percentage of total [3 H]phospholipid. Data represent mean $\pm$ SD of three independent experiments. Statistical significance was evaluated by Student's t test (* $p < 0.05$ compared to control)

Figure 2. Effects of various agents on PLD activity in M-1 cells. M-1 cells which had been cultured with (+Dexamethasone) or without (–Dexamethasone) 5 μ M dexamethasone were harvested and radiolabeled as described in "Materials and Methods". The cells were incubated in the absence or presence of 1 μ M dexamethasone, 10 μ M ATP, 1 μ M ionomycin, 1 μ M vasopressin, 1 μ M bradykinin, and 0.3 mM dibutyryl cAMP (cAMP) for 15 min. Data represent mean $\pm$ SD of three independent experiments. Statistical significance was evaluated by Student's t test (* $P < 0.05$; compared to no treatment, ** $P < 0.05$; compared to cells cultured without dexamethasone)

Figure 3. Dose-dependent effects of dexamethasone on PLD activity in M-1 cells. M-1 cells which had been cultured with or without 5 μ M dexamethasone were obtained as in Figure 2. Cells were incubated in the presence of the indicated concentrations of dexamethasone for 15 min. [3 H]PEth formation was measured as described in "Materials and Methods". Values are means $\pm$ SD of four determinations.

activity was further increased (from $1.40 \pm 0.06\%$ to $2.33 \pm 0.03\%$) and declined thereafter.

In cortical collecting duct, several agonists including vasopressin, bradykinin, ATP, and cAMP can activate transport machinery (Breyer and Ando, 1994). The effect of various agonists upon the activation of PLD was investigated and these results are presented in Figure 2. Stimulation of M-1 cells with ATP induced formation of [3 H]PEth with maximal activation occurring at 100 μ M. The effect of ATP on [3 H]PEth formation in cultured cells with dexamethasone was greater than in control untreated cells. It is well known that Ca^{2+} has a role in the activation of PLD, since Ca^{2+} ionophores stimulate PLD (Exton, 1997). However, ionomycin did not stimulate PLD activity significantly in M-1 cells. Dexamethasone-stimulated PLD activity was not further increased in presence of ionomycin and the activity was not changed by EGTA either (Figure 2 and data not shown). Taken together, these results suggest that Ca^{2+} does not appear to be responsible for PLD activation in M-1 cells. Vasopressin, bradykinin, and cAMP-increasing agent, dbcAMP were ineffective and simultaneous addition of these agents plus ATP or dexamethasone did not show any increase of PLD activity. The responses to dexamethasone and ATP were not augmented by PMA.

To distinguish between possible subtypes of PLD, we assessed the influence of ARF, PIP_2 and oleate on PLD activity of the cells. Simple cell permeabilization was used to analyze whether PLD could be activated by guanine nucleotide and ARF in intact cells. Digitonin

induces the formation of membrane pores enough to allow the passage of macromolecules, resulting in the depletion of cytoplasmic proteins. When the cells were permeabilized in the presence of guanine nucleotide, most of the GTP-binding proteins were also found to associate with membrane fraction. Figure 4 shows that there was a slight increase in [3 H]PEth formation when both GTP γ S and ARF were present, but this difference was not statistically significant. In our experiments, simultaneous addition of recombinant RhoA with ARF did not affect PLD activity in M-1 cells. High basal PLD activity that has been observed in cells cultured in the presence of dexamethasone was not decreased in digitonin-treated cells and addition of dexamethasone in assay medium had no effect on activity either (data not shown). These results suggest that GTP-binding proteins such as ARF did not stimulate PLD in M-1 cells. It is reported that a second form of PLD, PLD2, is constitutively active in the presence of PIP_2 (Colley *et al.*, 1997b). However, PLD activity in digitonin-treated cells was not changed by addition of PIP_2 . In contrast, there was 215% greater [3 H]PEth formation in the presence of digitonin and oleate than digitonin alone. To provide additional support for the presence of oleate-dependent PLD in M-1 cells, we measured PLD activity with various concentrations of oleate. As shown in Figure 5, [3 H]PEth formation was increased with millimolar concentrations of sodium oleate in the absence of digitonin. This dose-dependency coincides well with oleate-activated PLD in other intact cells or cell-free system (Massenburg *et al.*, 1994; Okamura and Yamashita, 1994; Lee *et al.*, 1998). Interestingly, oleate-induced [3 H]PEth formation in the

Figure 4. Effects of GTP γ S and oleate on PLD activity in digitonin-treated M-1 cells. M-1 cells cultured in the medium without dexamethasone were labeled with [3 H]myristic acid for 16 h. Cells (1×10^6) were preincubated with 10 μ M digitonin for 10 min and stimulated with 100 μ M GTP γ S plus 1 μ M ARF, 2 μ M PIP_2 , or 1 mM sodium oleate. PLD activity was measured as described in "Materials and Methods". Data represents mean $\pm$ SD of three independent experiments.

Figure 5. Dose-dependent effect of oleate on PLD activity in M-1 cells. M-1 cells cultured in the absence or presence of 5 μ M dexamethasone were radiolabeled as in Figure 4. The cells were incubated with indicated concentrations of sodium oleate for 15 min and measured formation of PEth. The data are represented as means $\pm$ SD of three independent experiments.

Figure 6. Expression level of PLD2 in M-1 cells. Confluent cells cultured in the absence (–) or presence (+) of dexamethasone were lysed in buffer containing 1% Triton X-100 and 1% deoxycholate for 1 h at 4°C. Equal amount of cell extracts (10 mg) were immunoprecipitated with anti-PLD2 antibody and protein A agarose. The supernatant fraction (lane 2) and beads (lane 3) obtained by immunoprecipitation were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and western blot. Lane 1 shows cellular extracts (100 µg).

cells was not affected by dexamethasone, suggesting that high basal activity in dexamethasone-treated cells may not be further activated by oleate. Taken together, these results demonstrate that enzyme responsible for the activity detected in M-1 cells is oleate-dependent PLD and PLD2 rather than PLD1.

We next studied PLD expression at the protein level using peptide specific antibody against PLD1 and PLD2. Both PLD1 and PLD2 were not detected in extracts of M-1 cells by direct blotting. Western blot analysis of the immunoprecipitates with anti-PLD2 antibody revealed a single band of protein migrating with an apparent molecular weight of 100 kD (Figure 6). When the expression of PLD2 in control and dexamethasone-treated cells was compared, the relative intensities of immunoreactive band were similar for both cells, indicating that dexamethasone had no apparent effect on PLD2 protein expression.

Discussion

Activation of PLD in mammalian cells is elicited by a wide variety of agonists in a variety of cell types. This report is the first to present evidence demonstrating PLD is stimulated by dexamethasone in the cortical collecting duct cell line, M-1. M-1 cells have been used as a model for studies of transport and signal transduction in the cortical collecting duct (Stoos *et al.*, 1991; Nakhoul *et al.*, 1998). Our initial experiments demonstrated an elevated basal PLD activity in M-1 cells cultured in the presence of dexamethasone compared to the untreated cells. [³H]myristic acid has been used successfully to radiolabel a phospholipid substrate pool that serves as a target for PLD. Treatment with the steroid did not

affect the extent of phospholipid labeling relative to control cells. To further elucidate this effect, we have evaluated PLD activity by addition of dexamethasone to the assay medium containing cells. Dexamethasone significantly increased [³H]PEth formation in both control and steroid treated cells and the change in activity after prolonged hormonal treatment coincides with that in short-term treatment, suggesting PLD activity is directly regulated by dexamethasone in intact cells. It has been also shown that dexamethasone significantly enhanced PLD activation by receptor agonists in retinoic acid-differentiated HL-60 cells but inhibited PMA-stimulated PLD activity (Collado-Escobar and Mollinedo, 1994). Transepithelial resistance and Na⁺ transport observed in cells cultured with dexamethasone was higher than in cells without dexamethasone (Nakhoul *et al.*, 1998). Although it is possible to speculate that there is a correlation between the PLD activation and the increase of transepithelial resistance by dexamethasone, the role of PLD on transporter activity is unknown. It has been reported that stimulation of principal cells, in cortical collecting duct, by vasopressin induced fusion of intracellular vesicles with the apical membrane leading to increased water permeability (Fushimi *et al.*, 1993; Nielsen *et al.*, 1995). Although PA derived from PC might directly stimulate some proteins to bind to membranes, it also stimulates phosphatidylinositol 4-phosphate 5-kinase to produce PIP₂ (Jenkins *et al.*, 1994). PIP₂ is reported to be an essential component in the process of membrane trafficking and the remodeling of cytoskeletal structure (De Camilli *et al.*, 1996; Martin, 1998). Therefore, it is possible that changes in the phospholipid membrane composition are elicited by dexamethasone-stimulated PLD and these changes affect the activity of an ion channel or a closely related protein.

Collecting duct function is finely controlled by different hormones (Breyer and Ando, 1994). When we measured PLD activity in response to agents that modulate function of M-1 cells, we found that ATP stimulated [³H]PEth formation. ATP exerts its effects via P2-purinoreceptors which have been shown to be coupled to PLD activation in a number of cell types (Boarder *et al.*, 1995). Treatment with dexamethasone or ATP showed a similar PLD activity pattern in both dexamethasone-treated and untreated cells. The activity was not changed by agents modulating intracellular Ca²⁺ concentrations. Dexamethasone has been reported to stimulate Na⁺ transport, however increased intracellular Ca²⁺ elicited with thapsigargin did not alter transport in M-1 cell (Nakhoul *et al.*, 1998). We observed no additivity between the ATP and dexamethasone effects. It has been reported that vasopressin induces increased intracellular cAMP levels leading to activation of ion channels (Fushimi *et al.*, 1993). However, vasopressin and other cAMP-increasing agents had no effect on formation of [³H]PEth, suggesting that PLD is not involved in

this process. Our results suggest that the response to steroid hormone and ATP is mediated through a PLD pathway in M-1 cells but the activation is independent of Ca^{2+} .

The mechanism(s) by which the PLD activity increases in dexamethasone-treated cells is still unclear. In order to understand the role and characteristics of hormone-sensitive PLD it is necessary to identify which isotype of PLD is present and activated in cells. PKC is one of the most potent stimulators of agonist-induced PLD activation, however we were unable to detect any activation of PLD by PMA at concentrations and incubation times shown to be maximal for other cells or tissues. The lack of PLD activity in response to PMA indicates that the PLD isotype in M-1 cell is not activated by PKC. There is evidence that PLD1 is the isoform regulated by PKC (Frohman *et al.*, 1999), however Siddiqi *et al.* recently suggested that PLD2 might also be regulated by PKC in intact cells (Siddiqi *et al.*, 2000). PLD1 activation is dependent on ARF and/or RhoA but PLD2 also can be activated by ARF in special circumstances (Frohman *et al.*, 1999). Our results demonstrate that cell permeabilization with digitonin did not reduce basal PLD activity and addition of myristoylated recombinant ARF failed to reconstitute PLD activation, suggesting that PLD in M-1 cells is independent of small GTP-binding proteins. As shown in Figure 6 we were able to detect PLD2 (but not PLD1) in M-1 cells by immunoblot of immunoprecipitated proteins. Although it is not yet clear whether mRNA levels correspond well to protein levels, the mRNA for both PLD1 and PLD2 could be detected in kidney (Meier *et al.*, 1999). It is possible that the expression of PLD1 is below the level of detection with the PLD1 antibodies used in our studies. It has been shown that regulation of cellular function by glucocorticoids involves protein synthesis. We have found that dexamethasone treatment did not affect significantly the expression of PLD and its regulatory factors such as ARF and RhoA in immunoblots. Although PLD2 expression appears to be unaltered by dexamethasone, PLD2 is likely to contribute to the high basal activity. Several different mechanisms might underlie the PLD2 stimulation such as covalent modification, cofactors or an increase in PIP_2 . However, we have not been able to detect any increase of activity by addition of PIP_2 . We can not rule out the possibility that PIP_2 may not be required for the activation of PLD 2 in intact cells. In addition to PLD1 and PLD2 a novel PLD activated by fatty acid has been described (Okamura and Yamashita, 1994). Although the nature of this oleate-stimulated PLD has still not been resolved, it is not activated by $\text{GTP}\gamma\text{S}$, ARF, or PIP_2 . Lee *et al.* reported that oleate-dependent PLD in L1210 (lymphocytic mouse leukemia cells) is independent of PMA (Lee *et al.*, 1998). Herein sodium oleate stimulated PLD activity similar to dexamethasone treatment, suggesting that oleate-dependent PLD plays a

role in M-1 cells. However, PLD activity in dexamethasone-treated cells was not further increased by oleate. Recently, Ryu and his colleagues reported that PLA2-mediated release of free fatty acid such as arachidonate and oleate stimulates PLD activity (Kim *et al.*, 1999b). Therefore, we speculate that dexamethasone induces accumulation of free fatty acids leading to upregulate PLD activity but its activity is not further enhanced by exogenous addition of oleate. Although we can not rule out the increased expression of oleate-dependent PLD or regulators of PLD2, we demonstrated that dexamethasone can directly affect activation of PLD.

In conclusion, the present study revealed that PLD2 and oleate-dependent PLD are both present in M-1 cells and the PLD activity is increased by dexamethasone treatment. The PLD activity in M-1 cells is modulated by exogenous ATP and by oleate. Further studies are necessary to elucidate the function of PLD in the regulation of cortical collecting duct cells.

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