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CHARACTERIZATION OF PHOSPHOLIPASE D ACTIVATION BY G_{M2} ACTIVATOR IN A *CELL-FREE* SYSTEM

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INDEXING WORDS

phospholipase D; G_{M2} activator; ADP-ribosylation factor

SYNOPSIS

Phospholipase D (PLD) activator which synergistically activates the enzyme with ADP ribosylation factor has recently been shown homologous to G_{M2} activator (Nakamura, S. et al.: Proc. Natl. Acad. Sci. USA 1998. 95, 12249/12253). The present studies were undertaken to further clarify the identity of the activator by immunological technique and to characterize the mechanism of activation of PLD by enzymological approach. The activator was further confirmed as G_{M2} activator by immunoblot analysis. Kinetic analysis showed V_{max} for the PLD reaction was 16 fold elevated by G_{M2} activator, whereas K_m for phosphatidylcholine remained constant by G_{M2} activator. These results strongly suggest that G_{M2} activator might activate enzyme by protein-

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protein interaction not by substrate modification. These results facilitate the understanding how the metabolism of both phospholipids and gangliosides is regulated by the same protein.

INTRODUCTION

Phospholipase D (PLD) has been implicated in a wide range of physiological and pathological processes including vesicular transport, cell motility, mitogenesis, oncogenesis, superoxide generation, and inflammation (for review, see[1]) and is activated by a wide variety of external signals (for review, see[2]). Mammalian tissues contain multiple PLD isoforms that are distinctly localized and regulated[1]. Biochemical studies have revealed that there are at least two groups of PLD in mammalian tissues. One is activated by small GTP-binding regulatory protein (small G-protein) such as ADP-ribosylation factor (ARF)[3, 4] and RhoA[5, 6], and the other is activated by sodium oleate[7, 8].

In earlier reports from this laboratory, a heat-stable factor from the cytosol of rat kidney, which synergistically activates rat kidney PLD with ARF and RhoA, has been reported[9]. This factor has recently been purified from rat kidney[10]. Amino acid sequence of the derived peptides from protease-treated activator was highly homologous to that of corresponding region of G_{M2} activator whose deficiency is known to result in AB-variant of G_{M2} gangliosidosis [11]. In the present studies, the PLD activator was further analyzed by immunological technique and the kinetics of PLD activation by G_{M2} activator was investigated.

MATERIALS

1,2-Di[1-^{14}C]palmitoyl-*sn*-glycero-3-phosphocholine (115 mCi/mmol) was purchased from DuPont-New England Nuclear. Phosphatidylethanol (PEt), a standard for TLC, was from Avanti Polar-Lipid, Inc. Guanosine 5'-*O*-(3-thiotriphosphate) (GTPγS) was from Boehringer-Mannheim. Glass-backed silica gel 60 was purchased from Merck. Other chemicals were of analytical grade.

METHODS

Preparation of recombinant human ARF1

Recombinant N-myristoylated human ARF1 was prepared from Escherichia coli expressing recombinant human ARF1 and human myristoyltransferase (a kind gift from Dr. R. A. Kahn, Emory University, Atlanta) as described by Randazzo and Kahn[12].

Preparation of PLD

PLD was partially purified from rat kidney as reported previously[11]. The partially purified PLD may similar or identical with PLD1 in that it is synergistically activated by ARF and RhoA[9].

PLD assay

PLD activity was assayed by measuring the formation of [^{14}C]PEt from [^{14}C]phosphatidylcholine ([^{14}C]PC) in the presence of ethanol and various activators including ammonium sulfate as described previously[13].

Other procedures

Heat-stable PLD activator was purified from rat kidney as described earlier[10]. Rabbit polyclonal antibody to a synthetic oligopeptide, Ser-Ser-Phe-Ser-Trp-Asp-Asn-Cys-Asp-Glu-Gly-Lys-Asp-Pro, which is a part of the deduced N-terminal-end region of human G_{M2} activator (amino acid residues 1-14)[14] was prepared as described earlier[15]. Immunoblot analysis was performed as described previously[15]. Protein was determined by the method of Bradford[16].

RESULTS

Immunological study

It has previously been reported from this laboratory that kidney PLD is synergistically activated by ARF and a heat-stable cytosolic protein from rat kidney[9]. Partial amino acid sequence of the heat-stable activator suggests that it is homologous to G_{M2} activator[11]. The activator was purified to near homogeneity (Fig. 1b) according to the method previously reported[10] and the identity of the activator was further verified by immunoblot analysis. An antibody against amino-terminal peptide of human G_{M2} activator specifically recognized the purified PLD activator, while preimmune serum failed to react it (Fig. 1a, lane 1 and 2, respectively) confirming that the activator is immunoreactive to anti-G_{M2} activator antibody. The heat-stable PLD activator is hereafter referred to as G_{M2} activator.

Activity of G_{M2} activator

The purified G_{M2} activator was tested for its ability to activate PLD. As shown in Fig. 2, PLD activity without any

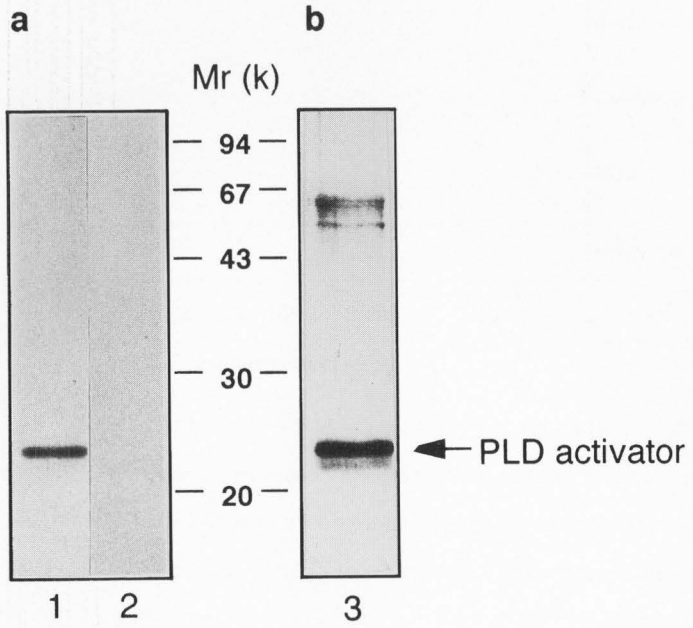

Figure 1. Immunoreactivity of PLD activator with G_{M2} activator-specific antibody. **a**, Purified heat-stable PLD activator (0.1 μg) was subjected to 12.5% SDS-polyacrylamide gel electrophoresis (SDS-PAGE), and then to immunoblot analysis. 1, with antibody against G_{M2} activator; 2, with preimmune serum. **b**, Purified heat-stable PLD activator (0.1 μg) was subjected to 12.5% SDS-PAGE, and stained with silver.

activators was almost undetectable. When ARF is present, the activity was strongly stimulated in consistent with previous reports[3, 4]. G_{M2} activator alone caused little or modest activation. However, both ARF and G_{M2} activator caused profound stimulatory effect which is consistent with previous reports[9-11].

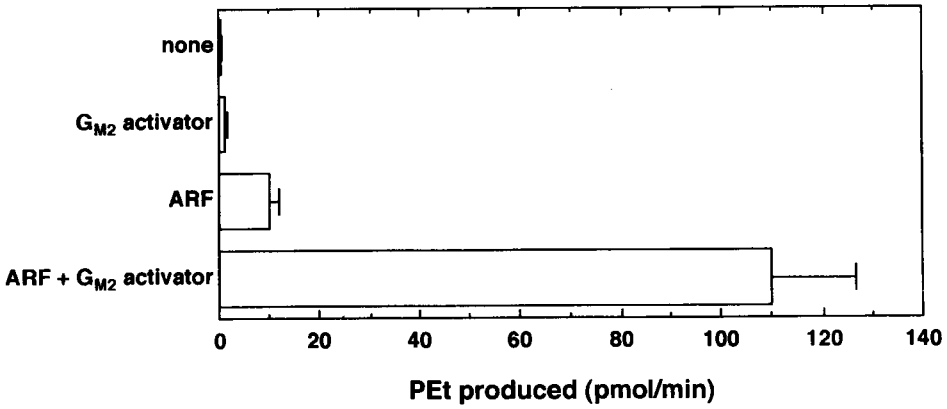

Figure 2. Reconstitution of PLD activity with ARF and G_{M2} activator. Partially purified PLD was assayed for enzyme activity under the standard assay condition as specified elsewhere[13] in the presence of various combinations of activators as indicated. Concentrations of ARF and G_{M2} activator used were 200 nM and 100 nM, respectively. Data represent the mean ± S.E. of 6 experiments performed in duplicate.

Kinetic analysis

Kinetic analysis of PLD activation by G_{M2} activator was studied next. As phosphatidylcholine (PC) increased, initial velocity of the enzyme reaction increased with a saturable manner in the presence or absence of G_{M2} activator (Fig. 3A). To determine the kinetic parameters more precisely, double reciprocal plot analysis was adopted. This kinetic analysis showed that the activation of ARF-dependent PLD by G_{M2} activator was mainly caused by increasing the V_{max} while the apparent K_m for PC was almost unchanged (Fig. 3B): the V_{max} values in the presence and absence of G_{M2} activator were 161 pmol/min and 10 pmol/min, while the K_m values for PC were 90 μM and 106 μM, respectively.

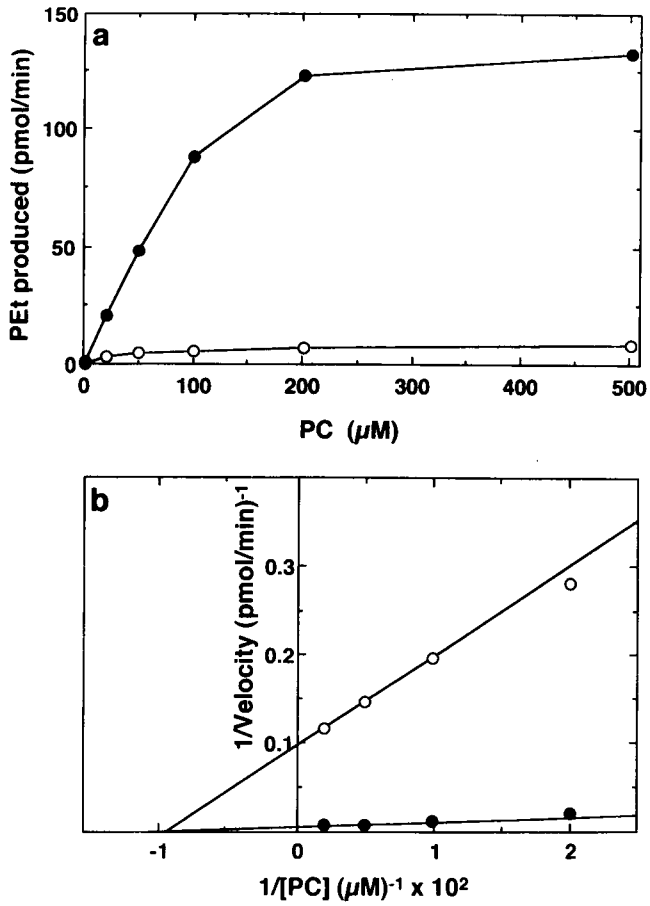

Figure 3. Characteristics of enhancement of ARF-dependent PLD activity by purified G_{M2} activator. **a**, Dependence of PC concentration on ARF-dependent PLD activation by purified G_{M2} activator. PLD activity was measured with 12.5 nCi [^{14}C]PC (115 mCi/mmol), various concentrations of dipalmitoylPC as indicated, and 200 nM ARF, 100 μM GTPγS, in the presence (●) or absence (O) of 100 nM of purified G_{M2} activator. **b**, Double-reciprocal plots of the results in Fig. 3A. ●, with G_{M2} activator; O, without G_{M2} activator. Data represent typical results from 5 experiments performed in duplicate.

DISCUSSION

Along with several evidences including heat stability[9], abundance in kidney[9], specific adsorption on octyl-Sepharose[10], and partial amino acid sequence of the peptides from protease-treated heat-stable PLD activator[11], immunoblot analysis performed in the present study further confirmed that the activator is indeed G_{M2} activator.

Initial interest in G_{M2} activator stemmed from the fact that the protein functions as a substrate-specific co-factor for β-hexosaminidase A and that its deficiency results in rare lysosomal storage disease, AB-variant form of G_{M2} gangliosidosis[17]. The mechanism of activation of β-hexosaminidase A reaction by G_{M2} activator is proposed to form a water-soluble complex of this protein with G_{M2} ganglioside which becomes a preferable substrate for β-hexosaminidase A[17]. The kinetic analysis in the present study indicates that G_{M2} activator increases the V_{max} of the enzyme reaction with the K_m value for PC being constant. It seems unlikely that G_{M2} activator forms complex with PC by an analogy to β-hexosaminidase A reaction, because the K_m value for PC remains unchanged by G_{M2} activator. G_{M2} activator might activate PLD by protein-protein interaction. Exact mechanism of PLD activation by G_{M2} activator, however, remains to be elucidated.

G_{M2} activator is now believed to be a lysosomal protein by its analogy to the distribution of β-hexosaminidase A. However, the majority (more than 80%) of G_{M2} activator in rat kidney could be recovered in the supernatant fractions after centrifugation at 100,000 x g for 60 min[10]. Further studies are necessary for

topological distribution of this protein in various tissues. Physiological relevance of G_{M2} activator in the regulation of PLD also remains to be clarified.

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