Volume 324, number 3, 349–352 FEBS 12614 June 1993

Receptor-binding capability of pancreatic phospholipase A_2 is separable from its enzymatic activity

Jun Ishizaki, Junji Kishino, Hiroshi Teraoka, Osamu Ohara and Hitoshi Arita

Shionogi Research Laboratories, Shionogi & Co. Ltd., 12-4, Sagisu 5-chome, Fukushima-ku, Osaka 553, Japan

Received 8 April 1993; revised version received 28 April 1993

Mammalian pancreatic phospholipase A_2 (PLA_2-I) has its specific receptor through which PLA_2-I induces a variety of biological responses. In this study, a fundamental relationship between the enzymatic and the receptor-binding activities of PLA_2-I was investigated. The specific binding of PLA_2-I to the receptor was found to be independent of Ca^{2+} which is requisite for the PLA_2 activity. On the basis of this observation, we designed and produced mutant PLA_2-Is without Ca^{2+}-binding abilities in order to demonstrate that the structural requirement for the enzymatic activity of PLA_2-I is not identical with that for its receptor-binding reaction. These mutant PLA_2-Is lost almost all enzymatic activity through a disturbance at the Ca^{2+}-binding site, as expected, but still retained a substantial affinity to the receptor, allowing us to conclude that the receptor-binding reaction of PLA_2-I is separable from its catalytic action.

Phospholipase A_2; Specific binding; In vitro mutagenesis

1. INTRODUCTION

Phospholipase A_2 (PLA_2; EC 3.1.1.4) catalyzes the hydrolysis of the fatty acid ester bond at the *sn*-2 position in phospholipids. Mammalian secretory PLA_2s so far identified are classified into two groups, groups I and II, on the basis of their characteristics in primary structure. The mammalian group I PLA_2 (PLA_2-I) is also called pancreatic PLA_2 due to its high abundance especially in pancreas. Although PLA_2-I has been considered to play a digestive role in mammals for a long time, recent studies show that this enzyme has a specific receptor on mammalian cells and elicits a variety of biological responses in a receptor-mediated manner [1–5]. The structural requirement for the receptor binding of PLA_2-I is proved to be very strict [3]; group II PLA_2s as well as non-mammalian group I PLA_2s are not recognized by the receptor, and furthermore, an enzymatically inactive precursor of PLA_2-I (proPLA_2-I) could not be a ligand even though the backbone structures of these PLA_2s are almost superimposable with that of mature PLA_2-I [6–8]. These facts raise questions as to what kind of structural properties of PLA_2 are crucial for the receptor-binding abilities and as to the ligand recognition mechanism of this receptor. As a first step to answering these questions on a molecular basis, we investigated the relationship between the enzymatic and receptor-binding activities of PLA_2-I and discriminated these in terms of Ca^{2+} requirement. A successful generation of an enzymatically inactive mutant PLA_2-I with a specific binding affinity to the receptor clearly indicates that the receptor binding and the enzymatic action are distinctive properties of PLA_2-I.

Correspondence address: H. Arita, Shionogi Research Laboratories, Shionogi & Co., Ltd., 12-4, Sagisu 5-chome, Fukushima-ku, Osaka 553, Japan. Fax: (81) (6) 458 0987.

Abbreviations PLA_2, phospholipase A_2; PLA_2-I, mammalian group I (pancreatic) phospholipase A_2; PCR, the polymerase chain reaction.

2. MATERIALS AND METHODS

2.1. *Measurements of receptor-binding affinity and enzyme activity*

The receptor-binding assay was performed as described previously [3] except that the bovine MDBK cell line (ATCC, CCL 22) was used as receptor expressing cells. The cells (1×10^6) were plated in culture dishes of 2.5 cm in diameter and grown in RPMI 1640 medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin. Binding affinities of recombinant PLA_2-Is were evaluated on their displacement potencies against the specific receptor binding of ^{125}I-labeled porcine PLA_2-I, and quantitatively expressed as the concentration of unlabeled PLA_2-I at which the half-maximal displacement is achieved, termed IC_{50} [3]. PLA_2 activities of recombinant PLA_2-Is were determined using [^{14}C]linoleoyl-phosphatidylethanolamine as a substrate [9].

For evaluating the effect of Ca^{2+} on the PLA_2 activity, the enzyme activity was measured in Hanks' solution (137 mM NaCl, 5.4 mM KCl, 0.34 mM Na_2HPO_4, 0.44 mM KH_2PO_4, 0.41 mM $MgSO_4$, 0.49 mM $MgCl_2$, 1.3 mM $CaCl_2$, and 5.6 mM glucose) in the presence or absence of 2 mM EDTA.

2.2. Construction of mutant PLA_2 expression plasmids

A site-specific mutagenesis of PLA_2-I cDNA was performed by an overlap extension technique reported by Ho et al. [10]. For introducing a mutation, mutagenic oligonucleotides were synthesized, oligonucleotides used for generating D49N mutant (Asp-49 to Asn) were 5′-GCTGCCAGACACATAACAAC-3′ (sense strand) and 5′-GTCATAGCAGTTGTTATGTG-3′ (antisense strand), and those for D49K mutant (Asp-49 to Lys) were 5′-TGCCAGACACATAAGAACTGC-3′ (sense strand) and 5′-GTCATAGCAGTTCTTATGTGT-3′ (antisense strand). Nucleotide residues underlined denote introduced mu-

tations. Using the human PLA_2-I expression plasmid pAM82-$HuPLA_2$ [11] as a template, 5′- and 3′-portions of PLA_2-I cDNA were separately amplified by the polymerase chain reaction (PCR) using the mismatch- containing oligonucleotides and oligonucleotides that hybridized at 5′ and 3′ ends of the cDNA. After the PCR, the amplified DNAs corresponding to the mutagenized 5′ and 3′-portions of PLA_2 cDNA, which have a sequence overlap at the mutagenized region, were purified on an agarose gel, mixed, and then subjected to PCR with the primers corresponding to 5′ and 3′ ends of the cDNA to produce a full length cDNA with site-specific mutation(s). cDNA fragments thus obtained were treated with Klenow fragment, digested with *Xho*I, and then inserted between *Xho*I and *Pvu*II restriction sites of pAM82. The expected sequence substitutions as well as the absence of artificial alternations in the cDNA structure during PCR were ascertained by sequencing the complete region of cDNA insert in the expression plasmid with a Sequenase sequencing kit version 2.0 (United States Biochemicals).

2.3. *Expression and purification of recombinant PLA_2-Is*

Transformation of *Saccharomyces cerevisiae* AH22 and culture of recombinant yeast were carried out as described by Kanda et al. [11]. Secreted $proPLA_2$-I in 700 ml of culture supernatant was absorbed to CM-cellulose CM52 equilibrated with 10 mM 4-morpholine ethanesulfonate buffer (pH 6.0) overnight at 4°C under gentle stirring. The $proPLA_2$ was eluted from the resin with a stepwise increase in NaCl concentration to 0.2 M in the same buffer described above, concentrated with Centricon 10 (Amicon), and activated through digestion with trypsin (10 μg/ml) in 50 mM Tris-HCl, pH 8.0 at 37°C for 30 min. The mature PLA_2-I thus obtained was purified by high-performance liquid chromatography (COSMOSIL, $5C_8$-300, 4.6 × 150 mm) with a linear gradient of acetonitrile from 15% to 55% in 0.1% of trifluoroacetic acid. The final purity of the recombinant protein was assessed by high-performance liquid chromatography and polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate, and its concentration was determined through amino acid analysis using a Hitachi amino acid analyzer (model 835).

3. RESULTS AND DISCUSSION

3.1. *Ca^{2+} dependence of the receptor binding*

First, we examined the requirement of Ca^{2+} in receptor-binding reaction; if the receptor-binding reaction is a Ca^{2+}-independent process, it would be a remarkable contrast to the catalytic action of PLA_2-I since PLA_2 activity is known to require a submillimolar concentration of Ca^{2+}. Fig. 1 shows that the removal of divalent cations by the addition of EDTA did not influence the binding ability of PLA_2-I whereas the enzymatic activity of PLA_2-I was almost lost under the same conditions, as expected. Since PLA_2-I undergoes some conformational changes upon Ca^{2+} binding [12], the results indicate that the Ca^{2+}-induced conformational alternation does not affect the receptor-binding reaction. This obvious difference in the Ca^{2+} dependence beween the receptor-binding and enzymatic activities means that these two properties are distinctive, and strongly suggests that the Ca^{2+} binding site is a possible target for generating a mutant PLA_2-I retaining the receptor-binding ability but losing its enzymatic activity. If we can successfully produce such a mutant PLA_2-I, this mutant provides us with solid evidence that the receptor-binding and enzymatic activities are separable properties of the PLA_2-I molecule. Furthermore, this mutant would serve as a

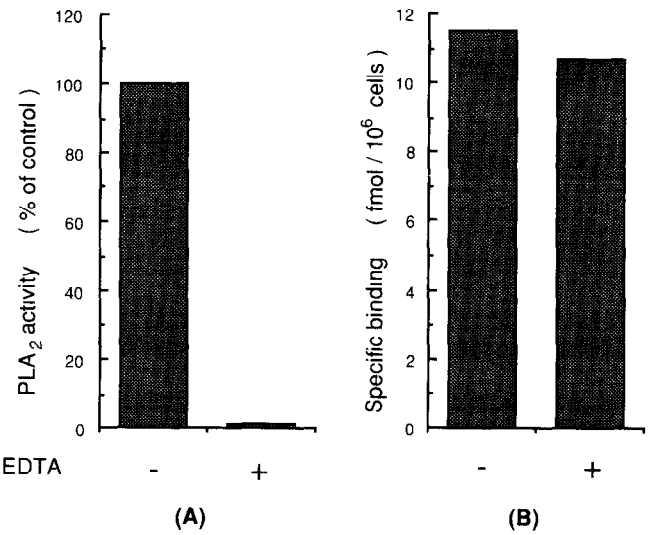

Fig. 1. Effects of Ca^{2+} on PLA_2-I enzymatic activity (A) and on its specific binding to PLA_2-I receptor (B). (A) 80 ng of human PLA_2-I was incubated with [^{14}C]linoleoyl-phosphatidylethanolamine (40 μM) in Hanks' solution at 37°C for 30 min in the presence (+) or the absence (−) of EDTA (final 2 mM). The control was defined as the activity in the absence of EDTA. (B) MDBK cells were incubated with 1 nM [^{125}I]PLA_2-I (porcine) in Hanks' solution at room temperature for 2 h in the presence (+) or absence (−) of EDTA, and then the specific binding was measured.

novel tool for analyzing the physiological roles of PLA_2-I and its receptor in a practical respect; this enables us to compare cellular events evoked by wild-type and these mutant PLA_2-Is and thereby elucidate which cellular events require PLA_2 activity for eliciting the receptor-mediated responses, if any. Therefore, we decided to pursue production of this type of mutant PLA_2-Is and examination of their properties.

3.2. *Receptor-binding and enzymatic activity of mutant PLA_2-Is*

From previous studies [13,14], it is now evident that Asp-49 in PLA_2-I is directly involved in Ca^{2+} binding. Van den Berge et al. examined the role of Asp-49 in porcine PLA_2-I using a site-specific mutagenesis technique and found that a mutant PLA_2-I with an amino acid residue substitution at Asp-49 to Lys or Glu lost the enzymatic activity almost completely through the reduction of Ca^{2+} binding ability [15]. This finding suggests that any amino acid residue replacement at position 49 probably causes inactivation of PLA_2-I due to lowering Ca^{2+} binding. Thus, we designed two mutants of human PLA_2-I expected to lose their Ca^{2+} binding ability; Asp-49 in the wild-type PLA_2-I was converted into Asn or Lys in a mutant, designated D49N or D49K, respectively. We produced these as well as the wild-type enzyme utilizing a recombinant expression system in yeast and they were purified to homogeneity by a combination of some chromatographic steps after activation of secreted $proPLA_2$-I with trypsin as de-

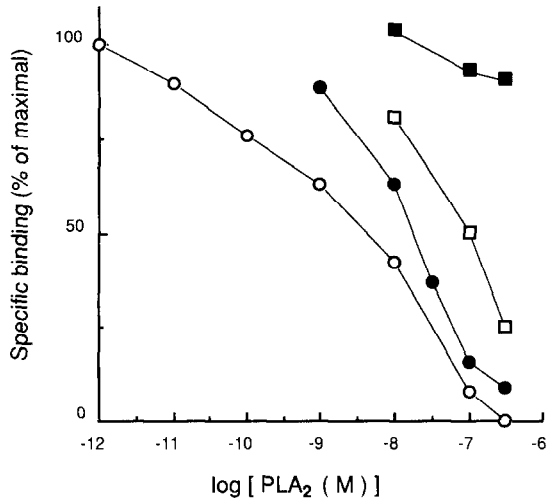

Fig. 2. Displacement assay of recombinant human PLA_2-Is. MDBK cells were incubated with ^{125}I-labeled PLA_2-I (porcine, 1.2 nM) and each recombinant unlabeled PLA_2-I (wild-type (○), or D49N (●), or D49K (□), or $proPLA_2$ (■)) at 4°C for 3 h. Bound radiolabeled PLA_2-I was measured as described previously [3]. The data represent the means of three independent experiments.

scribed in section 2. It is already proved that the recombinant wild-type PLA_2-I thus obtained is enzymatically active and indistinguishable from the native enzyme as far as examined [11].

First, we measured the binding affinities of the recombinant PLA_2-Is to the receptor by the displacement assay (Fig. 2). From the relationships between ^{125}I-labeled PLA_2-I displacements and added amounts of the recombinant PLA_2-Is, the binding affinities were estimated and quantitatively expressed as IC_{50} as shown in Table I together with their enzymatic activities. As expected, the enzyme activities of D49N and D49K were as low as that of $proPLA_2$-I (less than 1% of that of the wild-type PLA_2). However, in contrast to $proPLA_2$-I, the mutant PLA_2-Is certainly retained substantial receptor-binding ability; IC_{50}s of D49N and D49K were 3.8 and 20.8 times higher, respectively, than that of the wild-type PLA_2-I, although $proPLA_2$-I had virtually no binding ability to the receptor. These results indicate

Table I

Half-maximal inhibitory concentrations (IC_{50}s) and enzymatic activities of recombinant human PLA_2-Is

PLA_2-Is	Binding activity[a] IC_{50} value (M)	Enzymatic activity[b] (% of wild-type)
Wild-type	4.8×10^{-9}	100
D49N	1.8×10^{-8}	0.62
D49K	1.0×10^{-7}	0.51
$proPLA_2$	$>3 \times 10^{-7}$	0.47

[a] IC_{50}s were calculated from the displacement curves of the ^{125}I-labeled porcine PLA_2-I binding to the receptor as shown in Fig. 2.

[b] Enzymatic activities were measured using [^{14}C]linoleoyl-phosphatidylethanolamine as a substrate as described [9].

that disruption of a structural element required for the enzymatic activity is not necessarily accompanied with loss of the receptor-binding activity. In other words, the structure around the Ca^{2+}-binding site appeared not to be critical for the receptor-binding activity although the introduced structural perturbation at the Ca^{2+}-binding site by the mutation affects the receptor-binding ability to some extent. The decrease in the affinity of mutant PLA_2-Is to the receptor suggested that there is a possibility of sharing the structural motif(s) necessary for the enzymatic action and the receptor binding in part as anticipated from the behavior of $proPLA_2$-I. However, it can be unequivocally concluded that a Ca^{2+}-induced conformational change of PLA_2-I is not essential for receptor binding at all.

Although we do not know of differences in the actual three-dimensional structures of these mutants and the wild-type PLA_2-I in solution, it is very likely that the structure of the wild-type PLA_2-I is more suitable for both the enzymatic activity and receptor-binding than those of the mutants. In this respect, the fact that D49K retained a lower receptor-binding activity than D49N may be explained by the assumption that the substitution of negatively charged residue (Asp) in the wild-type PLA_2-I to a positive one (Lys) causes a larger perturbation to the overall PLA_2-I structure than that to a neutral residue (Asn).

A major concern in a series of physiological studies on the PLA_2-I-mediated responses is how to discriminate receptor-mediated consequences from a direct effect of the PLA_2 activity. To this end the mutant PLA_2-I (e.g. D49N) opens the way to examine cellular events evoked by PLA_2-I regardless of the enzyme activity and thereby makes it possible to gain a deeper insight to the PLA_2-I receptor-mediated reactions.

3.3. *Conclusions*

We successfully generated mutant PLA_2-Is which retained receptor-binding ability but lost enzyme activity by the destruction of the Ca^{2+}-binding site. Although it is difficult to conclusively define the region in PLA_2-I molecule responsible for the receptor-binding at present, these mutant PLA_2-Is gave us evidence that the requirements for the enzymatic and receptor-binding activities are not completely identical. In addition, these mutant PLA_2-Is would provide us with a powerful tool for further investigations on the signal transduction mechanisms of PLA_2-I mediated by the receptor.

Acknowledgements We thank A. Terawaki and A. Kanda of our laboratory for their technical assistance.

REFERENCES

[1] Arita, H., Hanasaki, K., Nakano, T., Oka, S., Teraoka, H. and Matsumoto, K. (1991) J. Biol. Chem. 266, 19139–19141.

[2] Hanasaki, K. and Arita, H. (1992) Biochim. Biophys. Acta 1127, 233–241

[3] Hanasaki, K. and Arita, H. (1992) J. Biol Chem. 267, 6414–6420.
[4] Kanemasa, T., Hanasaki, K. and Arita, H. (1992) Biochim. Biophys. Acta 1125, 210–214.
[5] Tohkin, M., Kishino, J., Ishizaki, J. and Arita, H. (1993) J. Biol. Chem. 268, 2865–2871
[6] Wery, J.P, Schevitz, R.W., Clawson, D.K , Bobbitt, J L , Dow, E.R., Gamboa, G , Goodson, Jr. T., Hermann, R.B., Kramer, R.M., McClure, D.B , Mihelich, E.D., Putnam, J.E., Sharp, J.D., Stark, D.H., Teater, C , Warrick, M.W. and Jones, N D. (1991) Nature 352, 79–82.
[7] White, S.P., Scott, D.L., Otwinowski, Z., Gelb, M.H. and Sigler, P.B. (1990) Science 250, 1560–1563
[8] Dijkstra, B.W., van Nes, G J.H., Kalk, K.H., Brandenburg, N P., Hol, W.G.J. and Drenth, J. (1982) Acta Cryst. B38, 793–799.
[9] Tanaka, K , Matsutani, S., Matsumoto, K. and Yoshida, T. (1992) J. Antibiotics 45, 1071–1078.
[10] Ho, S.N., Hunt, H.D., Horton, R.M., Pullen, J.K. and Pease, L.R (1989) Gene 77, 51–59.
[11] Kanda, A., Tamaki, M., Nakamura, E., Teraoka, H. and Yoshida, N. (1992) Biochim. Biophys. Acta 1171, 1–10
[12] Pieterson, W.A., Volwerk, J.J. and de Haas, G.H. (1974) Biochemistry 13, 1439–1445.
[13] Fleer, E.A M., Verheij, H.M. and de Haas, G.H (1981) Eur. J. Biochem. 113, 283–288.
[14] Dijkstra, B.W., Kalk, K H , Hol, W.G.J. and Drenth, J. (1981) J Mol Biol. 147, 97–123.
[15] van den Berge, C J , Slotboom, A.J , Verheij, H M. and de Haas, G H. (1988) Eur. J Biochem. 176, 353–357