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Identification of a membrane-associated 1-0-alkyl-2-arachidonoyl-*sn*-glycero-3-phosphocholine hydrolyzing phospholipase A_2in guinea pig 1 epidermis

Implications in the cutaneous biosynthesis of platelet-activating factor

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A membrane-associated 1-0-alkyl-2-arachidonoyl-GPC hydrolyzing phospholipase A_2 was identified in guinea pig epidermis. It is regio-specific (associated with the particulate microsomal fraction) and specific for the hydrolysis of 1-0-alkyl-2-arachidonoyl-*sn*-glycero-3-phosphocholine. It is sensitive to low calcium concentrations suggesting that it may be activated by increasing intracellular calcium. Since ether-linked phospholipids are known to exist in the epidermis, further understanding of the properties of this 1-0-alkyl-arachidonoyl-hydrolyzing PLA_2 may allow us to control the generation of 1-0-alkyl-2-lyso-*sn*-glycero-3-phosphocholine, a key substrate for the generation of the platelet-activating factor in the tissue.

Phospholipase A_2; Platelet-activating factor; Membrane enzyme; Epidermis

1. INTRODUCTION

Platelet-activating factor (1-0-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine) also known as paf-acether (PAF), is a potent autocoid which mediates allergic and inflammatory reactions [1,2]. Although its production was originally believed to be associated with a wide range of vascular cells such as: neutrophils, monocytes, vascular endothelial cells and the platelets [3–5], recent reports now indicate that human skin fibroblasts as well as epidermal cells can biosynthesize PAF [6,7].

The parent substrate for the biosynthesis of PAF is an ether-linked phospholipid, 1-0-alkyl-2-arachidonoyl-*sn*-glycero-3-phosphocholine (1-0-alkyl-2-AA-GPC). The hydrolysis of this 1-0-alkyl-2-AA-GPC by phospholipase A_2 yields two precursors of bioactive lipids: 1-0-alkyl-2-lyso-GPC and arachidonic acid (AA). The ether-linked lyso-GPC undergoes acetylation by the transfer of acetyl-CoA into the *sn*-2 position of the lyso-GPC by a specific acetyl-CoA: lyso-PAF acetyltransferase resulting in the formation of PAF [8] while the AA undergoes oxidative metabolism to yield a family of pro- and anti-inflammatory eicosanoids.

Since the rate-limiting step of this important biosynthetic pathway for PAF and AA (arachidonic acid) is the activation of a selective phospholipase A_2 (PLA_2), major efforts have been directed towards identifying a specific PLA_2 which is specific for the hydrolysis of the ether-linked parent substrate, 1-0-alkyl-2-AA-GPC. A variety of PLA_2 (notably, those that are calcium-dependent) have been described from diverse origins, [9]. A new family of PLA_2 recently been reported to exist in the cytosol of various tissues, cells including platelets [11]. The platelet PLA_2 has been purified to near homogeneity. It is reported to exhibit a molecular mass of about 90 kDa and it hydrolyzed phospholipids with an arachidonoyl residue more effectively than those with a linoleoyl residue. A cytosolic PLA_2 with a molecular weight of 100 kDa and with specificity for hydrolyzing 1-0-hexadecyl-2-arachidonoyl-GPC has been partially purified from a macrophage cell line RAW 264.7 [12]. This enzyme exhibits optimal activity at pH 8.0 and is calcium-dependent. More recently another cytosolic calcium-independent, plasmalogen-selective PLA_2 with a molecular weight of 40 kDa has been described in canine myocardial cytosolic fraction [13,14]. Similar phospholipases with basic pH optimum have also been described in rat lung [14] and in human amnion [16].

In contrast to the vast emerging information of cytoplasmic PLA_2 in a variety of tissues and cells, little is known about the existence and properties of membrane-associated PLA_2 which is selective for the hydrolysis of 1-0-alkyl-2-acyl-GPC. Since such a membrane-associated enzyme can be expected to be located within the cellular membrane of the cells where the phospholipid substrates do exist, we were prompted to explore whether such an enzyme exists in the guinea pig epider-

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mis where phospholipase A_2 activities have been described in the tissue and subcellular preparations from skin [16–20]. Enthusiasm for this exploratory effort was intensified by the recent identification in our laboratory of the existence in the epidermis of the alkylacyl- and alkenylacyl species of phosphatidylcholine and phosphatidylethanolamine as the most abundant of the three subclasses of phospholipids (diacyl-GPC/GPE, 1-0-alkylacyl-GPC/GPE and 1-0-alkenylacyl-GPC/PGE). Interestingly, these phosphoglycerides are highly enriched in the *sn*-2 position by polyunsaturated fatty acids (PUFAs) such as: *n*-6 PUFA: arachidonic acid (20:4*n*-6), *n*-3 PUFAs: eicosapentaenoic acid (20:5*n*-3) and docosahexaenoic (22:6*n*-3). The latter two *n*-3 PUFAs are elevated only after dietary intake. Thus, we present in the present paper, evidence of a membrane-associated arachidonoyl-hydrolyzing PLA_2 enzyme in the microsomal compartment of guinea pig epidermis.

2. MATERIALS AND METHODS

2.1. *Materials*

Male Hartley guinea pigs (400–450 g) were purchased from Simonsen (Gilroy, CA). [1-^{14}C]Arachidonic acid (S.A. = 55.6 mCi/mmol) was purchased from New England Nuclear Research Products (Boston, MA). Unlabeled arachidonic acid (5,8,11,14-eicosatetraenoic acid), snake venom phospholipase A_2 (lecithinase-A phosphatide-2-acyl-hydrolase), L-*a*-lysophosphatidylcholine, 1-0-hexadecyl-*sn*-glycero-3-phosphocholine (lyso-platelet activating factor), including cofactors: coezyme A and DL-dithiotreitol (DL-DTT) were purchased from Sigma Chemical Co. (St. Louis, MO). Adenosine-5′-triphosphate (ATP) was purchased from Boehringer Mannheim Biochemicals (Indianapolis, IN), and 5,8,11,14-eicosatetraynoic acid (ETYA) was purchased from Biomol Research Laboratories Inc. (Plymouth Meeting, PA).

2.2. *Preparation of guinea pig epidermal subcellular fractions*

Hair from the dorsum of normal male Hartley guinea pigs (400–450 g) was removed by shaving and depilation with sodium thioglycollate (Nair, Carter-Wallace, Inc.). After rinsing with deionized water, the animals were sacrificed by cervical dislocation and the skin rapidly removed. Epidermal strips (0.2 mm thick) were obtained using a Castroveijo electrokeratome (Storz, St. Louis, MO) and homogenized using a polytron (Brinkmann Instruments, Westbury, NY) in 10 vols. of ice-cold Tris-HCl buffer (20 mM), pH 7.4 containing 0.25 M sucrose and 1 mM EDTA to chelate endogenous calcium. The homogenate was centrifuged at 800 × *g* for 10 min at 4°C to remove the cell and nuclei debris. The resulting supernatant was centrifuged at 12,000 × *g* for 15 min to remove mitochondria. The resulting supernatant was finally subjected to ultracentrifugation at 100,000 × *g* for 60 min at 4°C in de Beckman L5-50E ultracentrifuge (Beckman Instruments, Palo Alto, CA) to give the high speed supernatant and microsomal fractions. The 100,000 × *g* supernatant fraction was concentrated to smaller volume and the pellet resuspended in 50 mM phosphate buffer, pH 7.4. Protein concentration was determined by the Lowry method with bovine serum albumin used as standard. The resuspended pellet and the supernatant fraction were used as sources of epidermal PLA_2 enzyme(s) for the subsequent incubations.

2.3. *Preparations of 1-0-alkyl-2-[^{14}C]AA-GPC and 1-acyl-2-[^{14}C]AA-GPC*

Radiolabeled 1-0-alkyl-2-[^{14}C]AA-GPC and 1-acyl-2-[^{14}C]AA-GPC were prepared by acylation of [^{14}C] arachidonic acid ([^{14}C](AA) and unlabeled arachidonic acid (50 nmol), into either 1-0-alkyl-2-lyso-GPC (50 nmol) or 1-acyl-2-lyso-GPC (50 nmol) in 1 ml of Tris-HCl buffer (50 mM) pH 7.4 containing: ATP (2.5 mM), $MgCl_2$ (10 mM), DTT (0.1 mM), coenzyme-A (CoASH) (0.1 mM), sucrose (0.35 M) and suspended rat liver microsomal fraction (350 μg protein). Incubation was for 15 min and the reaction was terminated by the addition of 4 volumes of $CHCl_3/CH_3OH$ (2:1, v/v). The extracted organic phase containing either the 1-0-alkyl-2-[^{14}C]AA-GPC or 1-acyl-2-[^{14}C]AA-GPC was purified by thin layer chromatography (TLC), using the solvent system: chloroform/methanol/acetic acid water (50:37.5:3.5:2, v/v/v/v). The respective radiolabeled bands were scraped off the plates, extracted with chloroform/methanol (2:1, v/v) and dried under N_2 gas. The purified labeled precursors were used as substrates for phospholipase A_2 assay.

2.4. *Hydrolysis of 1-0-alkyl-2-[^{14}C]AA-GPC and 1-acyl-2-[^{14}C]AA-GPC by guinea pig subfractions*

The standard assay for the GP PLA_2 activity included the incubation of the suspended particulate (microsomal) fraction of high speed cytosolic fraction (containing 600 μg protein) respectively with approximately 50,000 cpm of either 1-0-alkyl-2-[^{14}C]AA-GPC or 1-acyl-2-[^{14}C]A-GPC in 1.0 ml of phosphate buffer (50 mM) pH 7.4 containing: Ca^{2+} (0.05–2 mM) and eicosatetraynoic acid (ETYA) (50 μM). The incubation was carried out at 37°C for 20 min. For control experiments, the microsomal fraction was boiled for 30 min prior to incubations. For positive control, snake venom PLA_2 (25 μg) was used as enzyme source for incubations. The typical reaction mixture was terminated by the addition of 5 volumes of chloroform/methanol (2:1, v/v), the organic phase removed, dried under N_2 and the hydrolytic products separated by TLC using the solvent system: chloroform/methanol/acetic acid water (50:37.5:3.5:2, v/v/v/v).

3. RESULTS

3.1. *Hydrolysis of 1-acyl-2-[^{14}C]AA-GPC and 1-0-alkyl-2-[^{14}C]AA-GPC by high speed microsomal and cytosolic fractions*

The data shown in Fig. 1 revealed the distribution of phospholipase A_2 activities in both the cytosolic and particulate fractions with significant activity evident in the particulate fraction. Furthermore, the hydrolytic release of [^{14}C]AA from 1-0-alkyl-2-[^{14}C]AA-GPC was significantly ($P < 0.05$) higher than from 1-acyl-2-[^{14}C]AA-GPC in both cytosolic (Fig. 1A) and particulate (Fig. 1B) fractions. These results suggest that selectivety of substrate preferentially for 1-0-alkyl-2-[^{14}C]AA-GPC by a particulate PLA_2 exists in the GP epidermis.

3.2. *Effect of Ca^{2+} concentration on epidermal microsomal and cytosolic PLA_2 activity*

Since a role for CA^{2+} in arachidonoyl-hydrolyzing phospholipase A_2 has been suggested in a variety of reports, we determined whether or not the guinea pig PLA_2 activities expressed in the particulate and cytosolic compartments evolve from one or more enzymes. The data in Fig. 2B revealed that incubation of 1-0-alkyl-2-[^{14}C]AA-GPC with the particulate enzyme preparation in the absence of Ca^{2+} (chelated with EDTA) revealed a negligible basal activity. However, at the low extracellular Ca^{2+} concentration of 0.05 mM, approximately 58% of [^{14}C]AA was released from labeled substrate. This release of [^{14}C]AA increased rapidly and was maximal at 5 mM Ca^{2+} concentration (Fig.

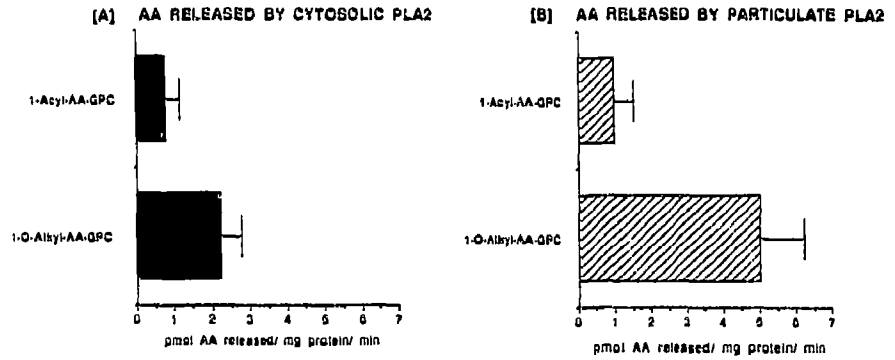

Fig. 1. Subcellular distribution of phospholipase A_2 activity in epidermal cytosolic and microsomal fractions. Suspended particulate (microsomal) fraction of the high-speed cytosolic fraction (containing 600 μg protein, respectively) was incubated with either 1-0-alkyl-2-[^{14}C]AA-GPC (50,000 cpm) or 1-acyl-2-[^{14}C]AA-GPC (50,000 cpm) in 1.0 ml of phosphate buffer (50 mM), pH 7.4, containing predetermined, maximal concentration of Ca^{2+} (0.05 mM) and ETYA (50 μM). The incubation was carried out at 37°C for 20 min. Reaction mixture was terminated by the addition of 5 vols. of chloroform/methanol (2:1, v/v). The organic phase was removed, dried and the hydrolytic products separated by TLC as described in the text. Phospholipase activity is expressed as pmol [^{14}]AA released, per mg protein, per min. The horizontal bars represent the mean ± S.E.M. of duplicate determinations from 3 separate experiments.

2B). Calcium concentrations higher than 0.5 mM inhibited the hydrolytic activity. In contrast, the incubation of the diacyl-GPC (1-alkyl-2-[^{14}C]AA-GPC) with the particulate enzyme at all concentrations (0.05–2 mM) resulted in minimal release of [^{14}C]AA when compared to the 1-0-alkyl-2-[^{14}C-AA]-GPC. This finding suggests that selectivity of the microsomal PLA_2 for the ether-linked phospholipid, (1-alkyl-2-acyl-GPC) exists in the guinea pig epidermis.

Incubations of the cytosolic enzyme preparation with the diacyl, 1-acyl-2-[^{14}C]AA-GPC and the ether-linked phospholipid, 1-0-alkyl-2-[^{14}C]AA-GPC (Fig. 2A) revealed moderate (15–20%) release of [^{14}C]AA from both labeled substrates. This cytosolic enzyme was not activated at Ca^{2+} concentrations (0.05–2 mM) tested.

3.3. *Kinetic properties of the microsomal PLA_2*

To gain more information on the preferential hydrolysis of 1-0-alkyl-2-[^{14}C]AA-GPC by the identified particulate PLA_2, we evaluated the effect of varying concentrations of both substrates on the hydrolytic release of [^{14}C]AA. As shown in Fig. 3, hydrolysis of the ether-linked phospholipid, 1-0-alkyl-2-[^{14}C]AA-GPC was markedly higher when compared to the diacyl 1-acyl-2-[^{14}C]AA-GPC. The double reciprocal plots (Lineweaver–Burke) of data from both experiments revealed that the apparent K_m values for 1-0-alkyl-2-[^{14}C]AA-GPC was 0.1 nmol AA/mg protein/min while that for 1-acyl-2-[^{14}C]A-GPC was 0.16 nmol AA/mg protein/min.

4. DISCUSSION

Data from these studies demonstrate the existence of a novel membrane-associated arachidonoyl-hydrolyzing PLA_2 in the guinea pig epidermis which preferentially hydrolyzes 1-0-alkyl-2-AA-GPC. Its activity is markedly elevated at low concentrations of Ca^{2+} and inhibited at higher concentrations. This property of marked activity at low Ca^{2+} concentrations suggests that the activity of this membrane PLA_2 may be regulated by the biochemical events of increased intracellular Ca^{2+}/protein kinase C emanating from the signal transduction process. Such a view is consistent with

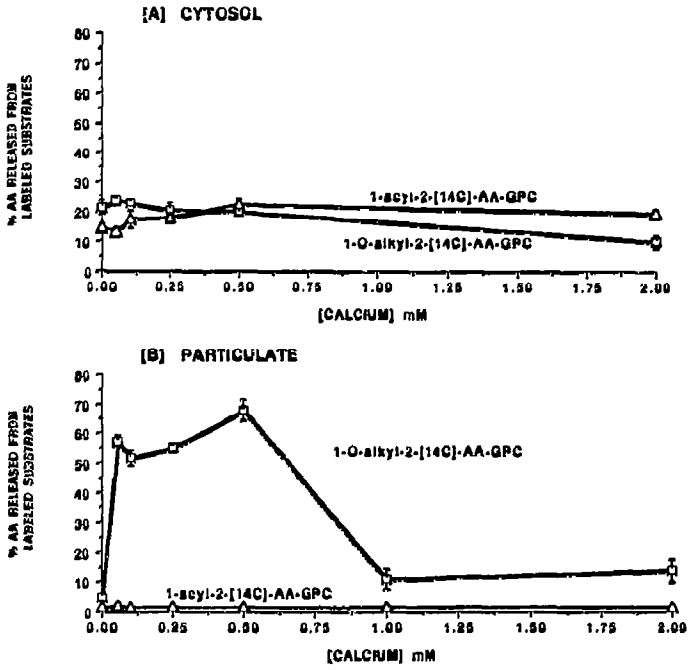

Fig. 2. Effect of Ca^{2+} concentration on epidermal microsomal and cytosolic PLA_2 activity. Epidermal strips were homogenized in the presence of 1 mM EDTA prior to fractionation into subcellular fractions. Each subfraction (particulate or cytosolic) was incubated with varying concentrations of calcium. Extractions and separation of metabolites are as indicated in the legend of Fig. 1. Each point represents the mean ± S.E.M. of duplicate determination from 3 separate experiments.

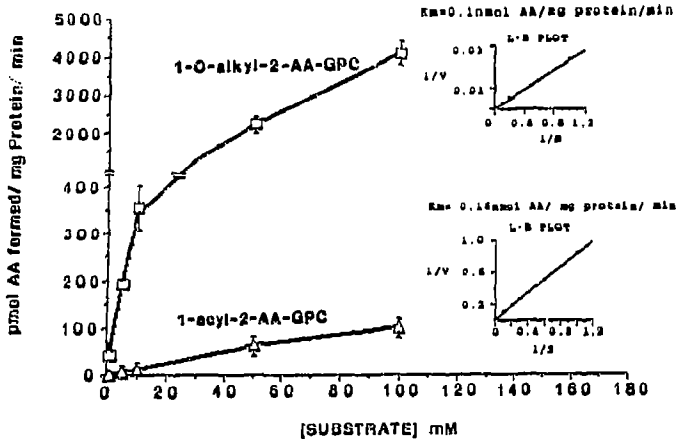

Fig. 3. Kinetic properties of the microsomal PLA_2. The suspended microsomal PLA_2 was incubated with varying concentrations of two substrates: the diacyl-GPC (1-alkyl-2-AA-GPC) and ether-linked phospholipid, 1-O-alkylacyl-GPC (1-O-alkyl-2-AA-GPC). Each point represents the mean ± S.E.M. of duplicate determinations from 3 separate experiments. The inserts show the results from the double reciprocal plots.

reports [23,24] that Ca^{2+} and protein kinase C (PKC) may regulate the biosynthesis of platelet activating factor.

In contrast to the vast number of cytosolic PLA_2 described in other cells and tissues, the particulate arachidonoyl-hydrolyzing PLA_2 described in these studies is regiospecific (microsomal) and substrate specific (will hydrolyze the alkyl acyl (1-O-alkyl-2-AA-GPC) and not the diacyl (1-alkyl-2-AA-GPC). Furthermore, unlike the microsomal PLA_2 reported in the rat lung [24], the guinea pig microsomal PLA_2 is extremely sensitive to low concentrations of calcium.

Although it has been reported that the stimulation of isolated human epidermal cells with Ca^{2+}-inophore (A23187) results in the production of PAF [7], the mechanism of this inductive generation of PAF is unknown. One possibility is that the microsomal, regiospecific arachidonyol-hydrolyzing PLA_2 for 1-O-alkyl-2-AA-GPC is activated after stimulation by Ca^{2+}-ionophore. This activation results in an inward flux of extracellular calcium, resulting in an increase in intracellular Ca^{2+} which then activates the microsomal phospholipase A_2. The consequence of this activation is the generation of PAF precursor (1-O-alkyl-2-lyso-*sn*-glycero-3-phosphocholine), its acetylation by the transfer of acetyl CoA into *sn*-2 position of the alkyl-2-lyso-GPC by the enzyme acetyl-CoA: 1-O-alkyl-2-lyso-*sn*-glycero-3-phosphocholine transferase and the formation of PAF. The activation of the microsomal PLA_2 by low Ca^{2+} concentrations described in these studies seem consistent with the described activation of PAF production by human epidermal cells. Further studies are, however, warranted to delineate this possible mechanism of action. Furthermore, elucidation of the properties of this novel epidermal microsomal arachidonoyl-hydrolyzing PLA_2 may allow us to regulate the hydrolytic release of substrates for the synthesis of pro-inflammatory lipid mediators.

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