Synthesis of cell-impermeable inhibitor of phospholipase A_2

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Phospholipase A_2 (PLA_2) in the cell surface membrane is considered a regulator of cellular secretion. The distinction between the role of the cell surface and the intracellular PLA_2 is not clear, since it has not been possible to differentiate unequivocally the activity of the enzymes in the various organelles. The use of an extracellular inhibitor of PLA_2 can greatly contribute to the understanding of cell surface PLA_2 function. In this paper, the preparation of a cell-impermeable inhibitor of PLA_2 is presented. This substance incorporates into lipid membranes and is capable of blocking the hydrolysis of membrane phospholipids by snake venom as well as by cell membrane PLA_2.

Phospholipase A_2 *Cell-impermeable inhibitor*

1. INTRODUCTION

Phospholipase A_2 (PLA_2, EC 3.1.1.4), which hydrolyzes phospholipids at the *sn*-2 position to produce a fatty acid and a lysophospholipid, is present in almost all cell types and subcellular fractions (review [1]). The activity of this enzyme has been correlated with various cell functions, such as exocytosis, platelet activation and prostaglandin production [2,3]. There is growing evidence that the enzyme involved is located in the cell membrane. Inhibitors of PLA_2 have been widely used in investigations of the role that cell membrane PLA_2 plays in secretion processes. However, since it is not clear whether these inhibitors are internalized, it is not possible to determine unequivocally that these inhibitors act exclusively at the cell surface [2,3]. Here, we aimed at preparing a PLA_2 inhibitor that would not penetrate into the cell. *N*-derivatives of phosphatidylserine (PS) have been shown to inhibit PLA_2 in cultured cells [4]. To prepare this material, we acylated the amino group of PS with the dicarboxylic fatty acid (dodecandioic acid) and linked the free carboxyl group to a high-M_r (M_r 70000) dextran. The derivatized PS, after binding to the carrier, maintained its ability to inhibit PLA_2.

2. EXPERIMENTAL

2.1. *Synthesis of the extracellular inhibitor of PLA_2*

2.1.1. Preparation of the PS dodecandioic derivative

300 μmol dodecandioic acid were added to 25 μmol PS dissolved in 2 ml dichloromethane, followed by 100 mg dicyclohexylcarbodiimide (DCCD), 0.2 ml triethylamine and 0.5 ml absolute methanol. The mixture was incubated for 24 h at 40°C. Analysis of the products by thin-layer chromatography (TLC) in chloroform/methanol/acetone/acetic acid/water (6:2:8:1) gave two spots sensitive to phosphate spray, at the origin and at $R_f = 0.75$, identified as PS and Ac-PS, respectively. This system was used for the purification of Ac-PS on preparative TLC plates. The product was characterized by NMR spectroscopy (300 MHz) in $CDCl_3/CD_3OD$ (2:1), and found to have a similar spectrum to PS with indication as to the presence of the additional dicarboxylic acid – two broadened triplets at 2.2 ppm belonging to the methylenic protons in α-position to the carbonyls and methylenic protons at 1.2 ppm, together with the proton of the fatty acid on *sn*-1 and *sn*-2. The phospholipid content of the acylated PS was measured by phosphate determination [5].

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2.1.2. Preparation of dextran hydrazide

10 g dextran-70 (M_r 70000) dissolved in 30 ml water were mixed with 0.6 g sodium periodate, stirred for 2 h and eluted through a 100 ml column of Dowex 1-X8-100 (chloride form) with distilled water. The oxidized dextran was then allowed to react with 300 mmol hydrazine hydrate at pH 5.0 and then reduced by adding 50 mmol sodium borohydride to the reaction mixture and bringing the pH to 9.0. After 10 h stirring, the dextran hydrazide was precipitated by the addition of absolute ethanol to the reaction mixture. The precipitate was dissolved in 60 ml distilled water, dialyzed extensively against water and lyophilized.

2.1.3. Binding of PS dodecandioyl to dextran hydrazide

200 mg DCCD were added to 10 μmol Ac-PS and 50 mg dextran hydrazide in 0.5 ml DMSO, followed by the addition of 200 mg DCCD, and stirred for 7 h at 45°C. The reaction mixture was then chromatographed on silica gel thin-layer plates in chloroform/methanol/acetone/water (6:2:8:1) and tested for phosphate (using the phosphate spray). The only positively stained material was at the origin, parallel to the location of dextran hydrazide, which was run as a marker, indicating that all the acylated PS was bound to the macromolecule. The dextran conjugate was precipitated and washed repeatedly with ethanol to remove DMSO and DCC. The precipitate was dissolved in distilled water and extensively dialyzed. The compound stained positively for sugar and phosphate. The PS content of the precipitate was measured by phosphate determination [5].

2.2. *Determination of the soluble PLA_2 activity*

The fluorescent phospholipid analogue, 1-acyl-2-(*N*-4-nitrobenzo-2-oxa-1,3-diazole)amino-caproylphosphatidylcholine (C_6-NBD-PC), purchased from Avanti (Birmingham, AL) was used as a substrate for snake venom PLA_2 (*Naja mucambiqa*, Sigma). In this substrate the fluorophore, NBD, is bound to caproic acid (C_6) at the *sn*-2 position of the phospholipid and PLA_2 activity can be determined by monitoring the production of the fluorescent fatty acid (C_6-NBD). Unilamellar vesicles composed of 40 mol% C_6-NBD-PC and 60 mol% dioleolylphosphatidylcholine (DOPC) were prepared by injection of an ethanolic solution into Tris buffer at pH 7.4 [6] and dialyzed against the buffer. The lipid vesicles were interacted with PLA_2 in the presence of 2 mM Ca^{2+} for the desired time and then subjected to lipid extraction and TLC as described by Sleight and Pagano [7]. The fluorescent fatty acid was extracted from the silica gel thin layer in chloroform/methanol/acid saline (1:1:5%) and its fluorescence intensity measured [7].

2.3. *Determination of cell-associated PLA_2 activity*

Cultured rat hepatocytes were incubated with serum-free Dulbecco's modified Eagle medium supplemented with lipid vesicles composed of C_6-NBD-PC and DOPC as described above. The cell culture was then collected using rubber policemen and subjected to lipid extraction, TLC and determination of C_6-NBD formation.

2.4. *Hepatocyte culture*

Rat liver cells were isolated by collagenase digestion and cultivated as primary monolayers on fibronectin coated plates as described [8]. The cells were used after 1 day of cultivation.

2.5. *Evaluation of the incorporation of the inhibitor into the lipid vesicle bilayers*

This was measured in vesicles composed of 80 mol% C_6-NBD-PC and 20 mol% DOPC. The NBD fluorescence is fully self-quenched and can be increased by dilution of the fluorescent phospholipid due to the insertion of other phospholipids [9,10]. The incorporation of the derivatized PS into the substrate vesicles was evaluated by the increase in NBD fluorescence intensity upon the addition of the inhibitor to the vesicle solution.

3. RESULTS AND DISCUSSION

The incorporation of Dex-Ac-PS into the C_6-NBD-PC/DOPC bilayer and its effect on C_6-NBD-PC hydrolysis were examined and compared with that of the unbound Ac-PS. Addition of Ac-PS, which had been dispersed in Tris buffer by sonication, to C_6-NBD-PC/DOPC vesicles (20 μM total lipid), produced an immediate increase in the fluorescence intensity of NBD, indicating that Ac-PS is readily incorporated into the

vesicle membrane. Maximal recovery of the fluorescence was obtained at an Ac-PS/C_6-NBD-PC ratio of 1:1 (at about 35 mol% NBD). A similar phenomenon was observed when Dex-Ac-PS was added to C_6-NBD-PC/DOPC vesicles, except that the concentration of the bound Ac-PS required for maximal fluorescence recovery was about 8-fold higher than with unbound Ac-PS. This difference is expected since the binding of Ac-PS to dextran increases its distribution in the aqueous phase.

The hydrolysis of C_6-NBD-PC by snake venom PLA_2 and its inhibition by both Ac-PS and Dex-Ac-PS are presented in fig.1. Interacting PLA_2 with the C_6-NBD-PC/DOPC vesicles gave only two fluorescent products, as identified by TLC: that of C_6-NBD-PC and C_6-NBD. The fluorescence quantum yield in the extraction solvent was the same for both the substrate and product, and full fluorescence recovery was obtained following the procedure, in either the absence or the presence of the inhibitors. Hence, the formation of C_6-NBD is a direct measure of PLA_2 action and the inhibitors do not activate alternative pathways.

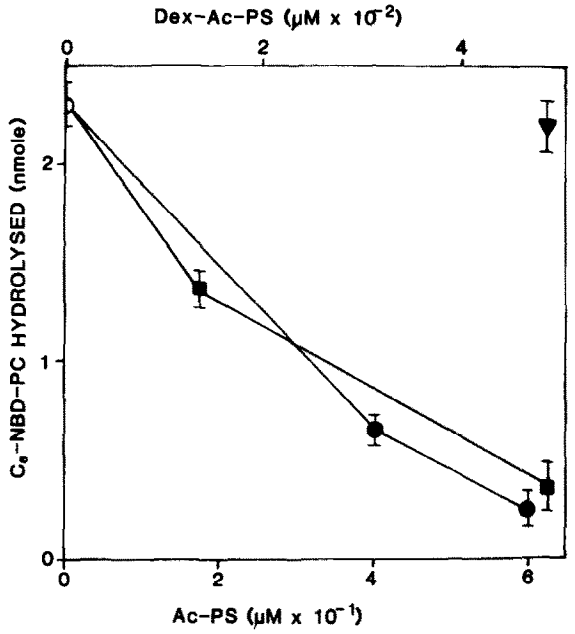

Fig.1. Effect of Ac-PS and Dex-Ac-PS on the hydrolysis of C_6-NBD-PC by snake venom PLA_2. 50 μM lipid vesicles composed of C_6-NBD-PC/DOPC at 2:3 molar ratio were incubated with 0.05 g/ml *N. mocambique* PLA_2 for 15 min at 37°C. The reaction mixture was then subjected to lipid extraction and TLC. C_6-NBD was extracted from the silica plate and its fluorescence was measured with reference to standard curves of known amounts of C_6-NBD. Each datum represents the mean ± SD for 3 replications. Incubation with (●) Ac-PS, (■) Dex-Ac-PS, (▼) dextran-hydrazide (without Ac-PS) in equivalent concentration to that of Dex-Ac-PS.

Fig.1 shows that the hydrolysis of C_6-NBD-PC was practically blocked when the concentration of the Ac-PS was about equal to that of the substrate. As expected from the distribution of Dex-Ac-PS between the aqueous phase and the lipid bilayer, the concentration of this inhibitor required for full inhibition was about 10-fold higher than that of the substrate. It should be noted that dextran-hydrazide (without the acylated PS) did not affect the hydrolysis of C_6-NBD-PC (fig.1).

The effect of the cell-impermeable inhibitor on PLA_2 in intact cell membrane was examined using cultured rat hepatocytes as described in section 2. The cells were incubated for 1 h with C_6-NBD-PC/DOPC vesicles as described in the legend to table 1, then washed and examined under a fluorescence microscope. The fluorescent lipid was visualized in the plasma membranes, but not in intracellular membranes, suggesting that the substrate incorporates and accumulates predominantly in the plasma membrane. Similar to the observation with liposomes and the soluble PLA_2 (fig.1), incubation of the cells with C_6-NBD-PC/DOPC vesicles produced only two fluorescent products: that of C_6-NBD-PC and C_6-NBD. This indicates that the fluorescent fatty acid formed by the cell membrane PLA_2 does not incorporate into other lipids. Thus, determination of C_6-NBD produced is a measure of the cell membrane PLA_2 activity. As shown in table 1, the hydrolysis of the incorporated C_6-NBD-PC by the membrane PLA_2 is effectively inhibited by increasing concentration of Dex-Ac-PS.

The attainment of the desired inhibitor required that the derivatized PS be capable of incorporation into the lipid bilayer and that its binding to high-M_r dextran would not prevent its incorporation into the membrane and not abolish its inhibitory capacity. These requirements are met by the preparation reported here. The long-chain dicarboxylic fatty acid fulfills two functions: derivatization of the PS amino group to form a PLA_2 inhibitor, and acting as a spacer between the PS in-

Table 1

Effect of Dex-Ac-PS on the hydrolysis of C_6-NBD-PC by liver cell membrane PLA_2

Treatment	C_6-NBD produced (FU/mg cell protein)
Control	472 ± 21
+ 50 μM Dex-Ac-PS	192 ± 23
+ 100 μM Dex-Ac-PS	54 ± 17
+ Dex-hydrazide (at mass concentration equivalent to 100 μM Dex-Ac-PS)	463 ± 27

Cells were incubated for 1 h at 37°C with lipid vesicles as described in the legend to fig.1, with increasing concentrations of Dex-Ac-PS. The cell culture was then subjected to lipid extraction, TLC and determination of C_6-NBD produced. Each datum represents the mean ± SD for 5 separate dishes of cultured hepatocytes. FU, arbitrary fluorescence units. 500 FU are equivalent to about 1.5 nmol C_6-NBD. The concentration of Dex-Ac-PS relates to the PS concentration as measured by phosphate determination

corporated into the lipid bilayer and the macromolecular carrier in the aqueous phase, aimed at preventing the internalization of the inhibitor into the cells. The results demonstrate that binding of Ac-PS to dextran does not prevent the incorporation of the derivatized PS into lipid membrane and that the hydrolysis of liposomal C_6-NBD-PC by soluble PLA_2, as well as the hydrolysis of C_6-NBD-PC incorporated into cell membrane by the membrane PLA_2, is inhibited with increasing concentration of Dex-Ac-PS. As already noted, *N*-derivatives of phosphatidylserine have been shown to incorporate into cell membrane, presumably into plasma, and inhibit cellular PLA_2[4]. The cell-impermeable PLA_2 inhibitor described here, which incorporates into lipid membranes, provides a novel means for differentially investigating cell surface PLA_2 and its role in cell function.

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