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A NOVEL BINDING ASSAY FOR PHOSPHOLIPASE A_2

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Abstract—We have devised a rapid and simple assay for estimating the binding of pancreatic phospholipase A_2 to a bilayer lipid membrane. The binding was observed to be extremely rapid at 37° and was absolutely dependent upon Ca^{2+}. Amongst several drugs known to inhibit the catalytic activity of phospholipase only mepacrine at high concentrations (500 μM) and chlorpromazine (100 μM) were active. Treatment of the enzyme with *p*-bromophenacylbromide did not inhibit binding. Several alcohols potentiated binding whereas detergents tended to inhibit. Amongst several purified proteins tested, only the steroid-induced anti-phospholipase protein lipocortin prevented binding. The use of this assay in screening for antiphospholipase agents is discussed.

Phospholipase A_2 (PLA_2, EC 3.1.1.4) specifically catalyses the hydrolysis of the 2-acyl ester linkage of 3-sn-phosphoglycerides in a Ca^{2+}-dependent reaction. A characteristic of soluble PLA_2s is the strong dependence of their catalytic properties upon the physical state of the substrate, such that the hydrolysis rate for substances in organised lipid–water interfaces is higher than when the same substrate is a molecularly dispersed solution.

To enable the catalytic event to occur, the phospholipase must first bind to its substrate in a specific fashion. The interaction between pancreatic PLA_2 and aggregated substrate occurs at a site functionally and topographically distinct from the catalytic site, termed the "interface recognition site" (IRS). This site, which surrounds the catalytic centre and consists of mainly hydrophobic residues, is present in the active enzyme but not its zymogen [1, 2].

Although the enzyme possesses this IRS, long chain diacyl phospholipids such as phosphatidylcholine are poor substrates for PLA_2, although they are more susceptible to hydrolysis at their phase transition temperature [3]. This and other findings have led to the concept that the "quality" of the lipid–water interface is important in determining the interaction between the enzyme and its substrate, and hence the hydrolysis rate [4]. The enzyme penetrates bilayers at sites of defect in lipid packing, such as those which occur at the phase transition temperature, or which can be introduced by sonication of the substrate [5, 6], by reaction products, i.e. lyso-phospholipid and fatty acids [7, 8] or by drugs such as alcohols which act as "spacer" molecules facilitating enzyme penetration [9].

The interaction of PLA_2 with lipid interfaces has been studied with a number of techniques, including UV difference spectroscopy [10], fluorescence spectroscopy [10], sephadex equilibrium filtration [1] and calorimetric methods [11]. These techniques have employed various well-characterised systems of pure phospholipids in order to identify features of both enzyme and substrate which are important for their interaction, and have demonstrated for instance the importance of the Ala-1 residue [12] and that alkylation of His-48, which destroys the catalytic activity of the enzyme does not affect its ability to interact with bilayers [1], although it may affect its ability to bind to mixed micelles [13].

Drugs may interfere with PLA_2 activity by interacting directly with the enzyme, or indirectly by affecting the physical state of the substrate and altering its binding [14, 15]. Thus since catalytic capacity is not essential for binding, a method which can measure binding alone may provide insight into the nature of drug action. In addition, the measurement of binding to a complex biological membrane may reflect more accurately the effect of a drug in a biological situation.

Hydrolysis of labelled membrane phospholipids of *E. coli* has been successfully used to assay the hydrolytic activity of PLA_2s from a variety of sources [16, 17]. We have adapted this technique and here describe a rapid and simple method to assess the binding of PLA_2 to autoclaved *E. coli*, and the effects of various substances upon this event.

MATERIALS AND METHODS

Human recombinant Lipocortin I was prepared as described by Wallner *et al.* [18] and was the gift of Biogen Research Corps Inc. Dexamethasone sodium phosphate was obtained from Organon Labs Ltd. (Cambridge, U.K.), heparin from Evans (Greenford, Middx, U.K.), *p*-bromophenacylbromide (*p*BPB), butanol, hexanol and benzyl alcohol from Fisons (Luton) and all other chemicals from Sigma (Poole, Dorset, U.K.). [^{125}I] was purchased from Amersham International (Amersham, Bucks, U.K.).

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Preparation of E. coli. *E. coli* AB1157 K-12 strain [19] were grown in standard culture conditions and autoclaved for 15 min at 121°. The cells were then washed repeatedly in 10 mM MOPS (3-*N*-Morpholino]propanesulfonic acid) buffer pH 7.5 and resuspended a final density of 10^{10}–10^{11} cells/ml in the same buffer containing 1 μg/ml sodium azide.

Labelling of PLA_2. Pig pancreatic PLA_2 (Sigma) was iodinated by a modified method of Hunter and Greenwood [20]. Briefly, 150 μg PLA_2 was incubated with 1 mCi [^{125}I] (100 mCi/ml) and Chloramine-T (1.5 μg) in phosphate buffer (100 mM) pH 7.0 for 10 min on ice. The reaction was stopped with sodium metabisulphite and residual [^{125}I] diluted with potassium iodide in phosphate buffer. Free iodine was separated from the iodinated protein using sephadex (G25) column chromatography, and the protein finally eluted in 10 mM MOPS buffer, pH 7.5. The iodinated enzyme was stored at 4° with sodium azide (1 μg/ml) as an antibacterial.

Assay conditions. Assays were routinely performed in 1 ml (final volume) 10 mM MOPS buffer pH 7.5 containing 1 mg/ml bovine serum albumin (BSA) and 10 mM Ca^{2+}. Aliquots of [^{125}I]-PLA_2 (5 ngs, approximately 20 × 10^3 cpm) were incubated with *E. coli* (10^8–10^9 cells) at 37° for 20 min, and bound enzyme removed by centrifugation at 10,000 *g* for 3 min. Aliquots of the supernatant were counted using conventional gamma-counting methods to assess "non-bound" enzyme. Specifically bound label was calculated as the difference in counts remaining in the supernatant in incubations with and without *E. coli*.

RESULTS

The binding of iodinated PLA_2 to *E. coli* was dependent upon the number of bacterial cells, and was inhibited by unlabelled PLA_2 with an ID_{50} of 100–500 ng PLA_2 (Fig. 1) depending upon the specific activity of the batches of iodinated enzyme.

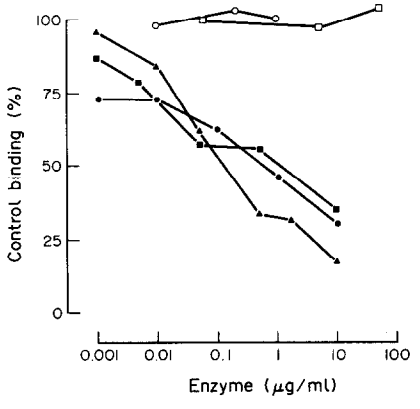

Fig. 1. Displacement of binding of labelled pig pancreatic PLA_2 to *E. coli* membranes by unlabelled (▲) pig pancreatic PLA_2; (■) *Naja mocambique* PLA_2; (●) bee venom PLA_2; (○) *B. cereus* phospholipase C; and (□) wheatgerm lipase. Each point is the mean of triplicates from one representative determination; standard errors omitted for clarity.

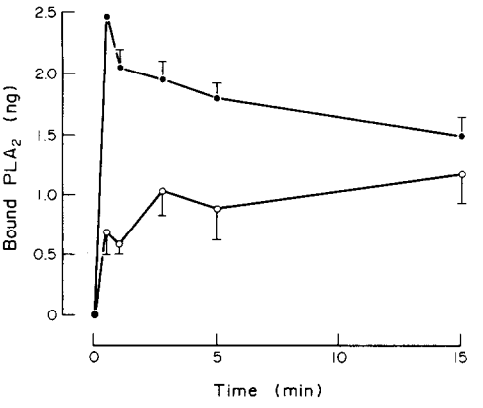

Fig. 2. Time course of binding of labelled PLA_2 to *E. coli* membranes at (○) 4° and (●) 21°. Each point is the mean ± SEM of 3 observations.

The phospholipases A_2 from snake and bee venoms also inhibited binding, but phospholipase C from *B. cereus*, and lipase from wheatgerm had no effect (Fig. 1).

Binding of PLA_2 occurred rapidly (Fig. 2), and the final extent of binding was not markedly temperature dependent: at 4° the final equilibrium binding was the same as that at 21°, although slightly slower in rate. The final binding appears to reflect an equilibrium, since addition of unlabelled PLA_2 after 15 min inhibited binding, although since a further 15 min incubation is required for full displacement (Fig. 3), it appears that the "off-rate" for the enzyme is much slower than the "on-rate".

Specific binding occurred only at pH > 6 with little observed at more acidic conditions. Little non-specific binding to the plastic tube occurred at the assay pH.

The binding of PLA_2 to *E. coli* was strictly Ca^{2+}-dependent, with a maximum at 5 mM. Sodium but

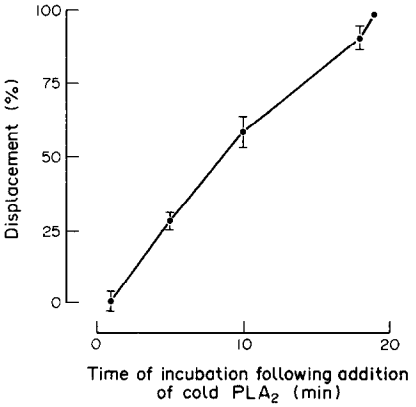

Fig. 3. The displacement, with time, of labelled PLA_2 from *E. coli* membranes following the addition of a 5000 fold excess of unlabelled PLA_2 added at time 0. Each point is the mean ± SEM of 3 observations.

Table 1. The effect of drugs and some other substances upon binding of labelled PLA_2 to *E. coli* membranes

Addition	Concentration	% Control binding ± SEM (N = 4)
p-BPB	50 μM	96.5 ± 2.8
Mepacrine	500 μM	65.3 ± 5.3*
Procaine	10 mM	106.2 ± 5.1
Lidocaine	10 mM	105.4 ± 2.9
Chlorpromazine	100 μM	20.3 ± 2.5*
Butanol	60 mM	157.5 ± 7.9*
Benzyl alcohol	20 mM	256.9 ± 6.2*
Hexanol	4 mM	246.9 ± 6.9*
Triton X-100	0.01%	32.1 ± 5.4*
Tween-20	0.01%	18.4 ± 9.1*
Deoxycholate	0.5 mM	162.5 ± 3.6*
Heparin	50 U/ml	98.4 ± 5.0

* $P < 0.05$.

not calcium EDTA (1 mM) totally abolished the binding which occurred without the addition of extra Ca^{2+}. No binding was seen when Mg^{2+} or Sr^{2+} (10 mM) replaced Ca^{2+} in the incubation medium. 10 mM Ba^{2+}, however, supported 50% of the binding seen with 10 mM Ca^{2+}. Na^+ and K^+ reduced Ca^{2+}-dependent binding at concentrations greater than 100 mM.

Pre-treatment of the iodinated enzyme with pBPB (50 μM) did not inhibit binding to *E. coli*, although the catalytic activity of unlabelled PLA_2 was destroyed by this procedure. In confirmation of this finding, pBPB-treated unlabelled enzyme successfully competed for binding with labelled PLA_2. Mepacrine inhibited binding of labelled PLA_2 to *E. coli* with an IC_{50} of approximately 600 μM. The effects upon binding of several other substances reported to affect PLA_2 activity are shown in Table 1. Butanol, hexanol and benzyl alcohol had biphasic effects, enhancing binding maximally at the concentration shown, but inhibiting binding at higher concentrations.

All assays were performed in the presence of 1 mg/ml fatty acid free bovine serum albumin, as preliminary experiments had shown that this reduced non-specific binding to the incubation tubes without affecting specific binding to *E. coli*. A variety of other proteins were tested in the presence of BSA to investigate their effects on the binding of the labelled PLA_2 to *E. coli* membrane (Table 2), and with one exception had no effect. This exception was human recombinant lipocortin [18], which strongly inhibited binding in a concentration-dependent manner with an IC_{50} of approximately 0.25 μM (10 μg/ml).

Table 2. IC_{50} values for inhibition of binding of labelled PLA_2 to *E. coli* membranes (all assays were performed in the presence of 1 mg/ml BSA)

Protein addition	IC_{50} Values μg/ml	M
Lipocortin	1–10	25–250 nM
Ovalbumin	≫1000	≫20 μM
Human serum albumin	≫1000	≫15 μM
Haemoglobin	>1000	>20 μM
Trypsin inhibitor	≫1000	≫50 μM
Metallothienin	≫1000	≫100 μM
Carbonic anhydrase	>1000	>30 μM
Rat serum	0.1–0.5%	—
Egg yolk lipoprotein	2–5%	—

Rat (and rabbit) sera were found to increase non-specific binding and also to inhibit specific binding with an IC_{50} value of 0.1–0.5%. Heat treatment (50°, 10 min) of serum did not affect its inhibitory activity. Egg yolk lipoprotein had a similar effect to serum with an IC_{50} against specific binding of 2–5%. The inhibitory effects may be due to the presence of alternative binding substrates for PLA_2 (Vadas *et al.* [21] have described the anti-phospholipase properties of serum as being largely due to albumin) and may thus illustrate a possible artefact to be taken into account when studying the inhibitory activity of crude biological extracts on PLA_2 binding.

DISCUSSION

Iodinated PLA_2 binds to *E. coli* membranes with characteristics similar to those displayed for binding to, or hydrolysis of, other substrates. The binding is Ca^{2+} dependent although Ba^{2+} can substitute to a certain extent. The Ca^{2+}-binding of PLA_2 has been thoroughly investigated [12], although it is not clear as to whether Ca^{2+} is essential for binding to all forms of substrate. For example Verheij *et al.* [22] showed Ca^{2+} to be essential for enzyme binding to monomeric lipids, but Goormaghtigh *et al.* [23] demonstrated binding to micelles in the presence of EDTA. We find some binding to *E. coli* membranes in the absence of added Ca^{2+}, but since this is abolished by $Na^+ \cdot$EDTA but not by $Ca^{2+} \cdot$EDTA, we attribute this to the presence of *E. coli*-associated Ca^{2+} as discussed by Elsbach *et al.* [24]. Pieterson *et al.* [25] have shown that Ba^{2+} is much less effective than Ca^{2+} at causing the characteristic changes in the UV spectrum of PLA_2: we cannot explain why Ba^{2+} should appear to be 50% as effective in this assay, although this may reveal a difference between those factors necessary for binding and those required for catalytic activity.

Jain and co-workers have reported that PLA_2 binds to discrete sites in phospholipid bilayers associated with defects in lipid packing [7, 8, 15, 26]. We do not know whether such sites are present in the *E. coli* preparation used, although Elsbach *et al.* [24] have shown that native *E. coli* are resistant to hydrolysis by PLA_2, and that autoclaving increases their susceptibility to the enzyme. This may be the result of destruction of their outer coat [27] allowing access of the enzyme to their lipid membrane. We noted in early experiments that the maximum binding to *E. coli* varied from batch to batch, and this may have been related to varying autoclaving protocols.

We have never observed total binding of the labelled PLA_2 to *E. coli* although bound label could be displaced by unlabelled PLA_2. An approximate 10-fold excess of unlabelled enzyme was required, however, suggesting that the labelled PLA_2 was not saturating available binding sites, presuming that

iodination did not alter the affinity of PLA_2 for its substrate. The maximum extent of binding varied between batches of labelled enzyme, which may reflect a varying degree of damage done to the enzyme during the iodination procedure resulting in "non-binding" enzyme molecules; the presence of albumin will also contribute alternative sites for PLA_2. Preliminary experiments indicated that the labelled enzyme retained catalytic activity, suggesting that the enzyme had not been severely damaged by the iodination procedure.

The enzyme binds very rapidly to *E. coli* membranes, and this is also true for binding to lipid bilayers [26]. The "off" reaction, however, appears to be slower, such that competition of bound PLA_2 by unlabelled enzyme requires 15–20 min for full displacement. This may be important when deciding the order in which potentially inhibitory substances are added to the reaction mixture. Treatment of the enzyme with pBPB destroys its catalytic activity due to phenacylation of the His-48 residue [28]. However, Pieterson *et al.* [1] reported that this procedure did not affect the ability of the enzyme to bind to lipid micelles. We find similar results with binding to *E. coli* membranes, and that the phenacylated enzyme is equipotent with control enzyme at displacing labelled PLA_2. In contrast, Nalbone *et al.* [13] found that pBPB prevented pig pancreatic PLA_2 from binding to mixed micelles of egg yolk lipoprotein and bile salts, although the addition of reaction products reversed this effect. This apparent discrepancy may reflect differences in substrate configuration.

We wished to confirm that our assay reflected true PLA_2-substrate binding, and investigated the effects upon binding of various drugs which have been reported to affect PLA_2 catalytic activity or binding in a variety of assays. We found that a number of "membrane-active" compounds had marked effects, suggesting that modulation of *E. coli* membranes could affect the enzyme interaction. Alcohols have biphasic effects upon PLA_2-catalysed hydrolysis of lipid bilayers and also in our assay on binding, first causing stimulation of hydrolysis or binding followed by inhibition at higher concentrations [5, 29]. The potency of the alcohol is related to its lipid solubility, and it is believed that they may act as spacer molecules facilitating penetration of the bilayer by the enzyme, or affect the phase behaviour of the lipid to increase the number of binding sites. Chlorpromazine also has membrane-active properties and in addition may inhibit Ca^{2+} and calmodulin mediated events [30, 31]. Chlorpromazine was a potent inhibitor of binding, and this could not be overcome by increasing the ambient Ca^{2+} concentration, suggesting its actions are lipid-mediated. Local anaesthetics have also been reported to inhibit PLA_2 in some assays [32, 33] but were without effect in this assay. Detergents generally stimulate PLA_2 activity, probably by dissolving substrates to make mixed micelles which are more susceptible to enzyme attack. We found that deoxycholate stimulated binding, but Tween-20 and Triton X-100 inhibited binding. This latter effect may be the result of the partial solubilization of the *E. coli*, and therefore not related directly to the binding event.

Mepacrine has been reported both to affect substrate structure and to interact directly with PLA_2 [14, 15]. Mepacrine inhibited binding of PLA_2 to *E. coli* at concentrations higher than those reported to inhibit the catalytic activity of PLA_2 of *in vivo*, but similar to those seen *in vitro* [14, 34]. Indomethacin, which has been reported to inhibit some phospholipases [35, 36] and dexamethasone which inhibits the enzyme indirectly had no effect at the concentration tested.

We found that non-specific binding of label to incubation tubes was reduced by the inclusion of albumin in the incubation buffer. A range of proteins tested in the presence of BSA had little effect upon specific binding, although complex lipid-containing mixtures such as serum or egg yolk did interfere with the assay by increasing non-specific binding and inhibiting specific binding: the inclusion of appropriate controls is necessary when the substances are present in reaction mixtures. One protein which did inhibit specific binding was the steroid-inducible antiphospholipase protein lipocortin, here used in the human recombinant form [18]. Lipocortin displays antiphospholipase activity in a number of whole cell systems and is also active against the isolated enzyme in several different systems [37]. The *E. coli* hydrolysis assay is particularly sensitive to the inhibitory action of the protein perhaps because of the presence of "physiological" bilayer lipid membrane (the gross morphology of the *E. coli* cells seems little changed by the autoclaving). The result obtained here suggest that the inhibitory action of lipocortin on phospholipase A_2 may be caused, not by an action at the catalytic site, but by hindering the initial interaction of the enzyme and substrate and preventing the formation of the E–S complex.

Because lipocortin was such a potent inhibitor of the binding event and this assay is relatively unaffected by the presence of other proteins, it could be extremely useful for monitoring the biological activity of column effluents or other biological fluids containing putative inhibitory proteins (this subject will be dealt with more fully in another paper), although this assay would obviously not detect substances which may affect catalytic activity without altering binding to the substrate.

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Note added in proof

Haigler *et al.* (*J. biol. Chem.* **262**, 6921–6930, 1987) have now shown that [^{125}I]-lipocortin I (purified from human placenta) binds to *E. coli* membranes but not to pig pancreatic phospholipase A_2. Hence it appears likely that the action of human recombinant lipocortin I reported here could occur following interaction with the *E. coli* membranes, preventing binding of [^{125}I]–PLA_2 to its substrate.