Inhibition of human cytosolic phospholipase A₂ by human annexin V

Andrew G. BUCKLAND and David C. WILTON¹

Department of Biochemistry, University of Southampton, Bassett Crescent East, Southampton SO16 7PX, U.K.

The ability of annexins, particularly annexin 1 (lipocortin 1), to inhibit phospholipase A₂ (PLA₂) is well known and a substrate depletion mechanism is now widely accepted as the explanation for most inhibitory studies. However, there are only a very limited number of reported studies involving annexins and the high-molecular-mass cytosolic PLA₂ (cPLA₂). In this study we have examined the effect of human recombinant annexin V, a potentially abundant cytosolic protein, on the ability of human recombinant cPLA₂ to hydrolyse a variety of phospholipid substrates. The results show clearly that, under the conditions of our study, annexin V can inhibit cPLA₂ activity by a mechanism of substrate depletion and that this inhibition is dependent on the nature of the phospholipids and the concentration of Ca²⁺ ions in the assay. The hydrolysis of 1-stearoyl 2-arachidonyl phosphatidylcholine by cPLA₂ was not significantly affected by annexin

V over a range of Ca²⁺ concentrations (1 μM–2.5 mM), a result that presumably reflects the zwitterionic nature of the phospholipid and the known inability of annexins to bind to such interfaces. In contrast, the hydrolysis of dioleoyl phosphatidylglycerol, which is an effective anionic phospholipid substrate for this enzyme, and more significantly that of 1-stearoyl 2-arachidonyl phosphatidic acid, were readily inhibited by annexin V, although these effects were Ca²⁺-dependent. The Ca²⁺ concentrations required for inhibition in the assay system *in vitro* are greater than those associated with Ca²⁺-stimulated events within the cell, suggesting that a role for annexin V in regulating cPLA₂ activity might not involve a substrate depletion mechanism *in vivo* unless factors in addition to Ca²⁺ and phospholipids contribute to the binding of annexin V to cell membranes.

INTRODUCTION

The discovery of the lipocortins, identified by their being inhibitors of phospholipase A₂ (PLA₂) [1,2], provided some of the first structural and functional information that led to the identification of a large group of proteins now known as the annexins [3]. Both annexins and phospholipases A₂ are characterized by their ability to bind to phospholipid interfaces as the presumed first step in their biological action. The annexins have an absolute requirement for Ca²⁺ for this interfacial binding, whereas the two most well-defined classes of PLA₂, the 14 kDa secretory PLA₂ and the 85 kDa cytosolic PLA₂ (cPLA₂) also have a requirement for Ca²⁺ to allow overall function. In the case of cPLA₂, Ca²⁺ is not required for catalysis but is normally required for binding to biological membranes. This requirement is associated with an N-terminal Ca²⁺-dependent phospholipid-binding domain (CaLB) that is similar to several other proteins involved in signal transduction [4,5]. Ca²⁺ concentrations in the micromolar range promote translocation to membranes and are correlated with Ca²⁺ concentrations required *in vitro* for catalytic activity. Thus intracellular Ca²⁺ concentration, together with enzyme phosphorylation [4,5], is believed to have a crucial role in determining the catalytic activity of cPLA₂ within the cell and, in particular, the release of arachidonic acid. Therefore a complete understanding of factors within the cell that might regulate cPLA₂ activity is of considerable importance for many aspects of cell regulation.

The annexins show a marked preference for binding to interfaces, bilayers or vesicles containing anionic lipids, whereas for annexin V a specific receptor site for phosphatidylserine has been proposed [6]. No significant binding of annexins to pure phosphatidylcholine can be observed except possibly at very high

Ca²⁺ concentrations [7–9]. Although cPLA₂ does not have an absolute specificity for a particular class of phospholipid and most studies *in vitro* have been performed with 1-stearoyl 2-arachidonyl phosphatidylcholine (SAPC) or 1-palmitoyl 2-arachidonyl phosphatidylcholine, the substrate *in vivo* for this enzyme remains to be established clearly and therefore whether annexins could have a role in modulating enzyme activity by a substrate depletion mechanism. Annexin V has a high intracellular concentration, where it can form up to 2% of total cell protein in certain tissues [10]. Therefore, considering that both annexin V and cPLA₂ are found within the cytosol and have both been demonstrated to translocate to the nuclear membrane on increases in intracellular Ca²⁺ levels [11–13], it is of importance to investigate mechanisms by which annexin V could modulate cPLA₂ catalytic activity and, in particular, whether a substrate depletion mechanism could operate successfully in the cell.

In this study we have investigated the ability of human recombinant annexin V to inhibit human cPLA₂ *in vitro* by using a variety of substrates and monitoring cPLA₂ activity with a continuous fluorescence displacement assay.

EXPERIMENTAL

Dioleoyl phosphatidylglycerol (DOPG), 1-stearoyl 2-arachidonyl phosphatidic acid (SAPA), arachidonic acid and oleic acid were obtained from Sigma. SAPC was purchased from Avanti Polar Lipids (Alabaster, AL, U.S.A.). The purity of all lipids was confirmed by TLC. 11-Dansylaminoundecanoic acid was obtained from Molecular Probes (Eugene, OR, U.S.A.). Recombinant human cPLA₂, which was a gift from Zeneca Pharmaceuticals (Macclesfield, Cheshire, U.K.), was purified as described

Abbreviations used: CaLB, Ca²⁺-dependent phospholipid-binding domain; (c)PLA₂, (cytosolic) phospholipase A₂; DOPG, dioleoyl phosphatidylglycerol; SAPA, 1-stearoyl 2-arachidonyl phosphatidic acid; SAPC, 1-stearoyl 2-arachidonyl phosphatidylcholine; SUV, small unilamellar vesicle.

¹ To whom correspondence should be addressed.

previously [14]. Recombinant human annexin V was a gift from Dr. J. Romisch (Behring Institutes, Marburg, Germany). N-terminal sequencing confirmed that the recombinant annexin V, in contrast with the native protein, has an unblocked N-terminal alanine residue; however, comparisons between the two proteins have given identical results [15]. Fatty acid-binding protein was prepared as previously described [16]. The purity of all proteins was confirmed by SDS/PAGE.

PLA₂/annexin V assays

A continuous assay of fluorescence displacement, which has been described previously [17], was used to monitor cPLA₂ and annexin V activity. Assays were performed in a 1 ml volume in plastic cuvettes containing 0.1 M Tris/HCl, pH 8.0, 0.1 M NaCl, CaCl₂ (for concentrations see the legends to the Figures), 1 mM EGTA, 11-dansylaminoundecanoic acid (1 μ M), fatty acid-binding protein (approx. 12 μ g) and phospholipid substrate (generally 2.5 nmol/ml) in the form of small unilamellar vesicles (SUVs). Phospholipid was added to the assay cocktail either by rapid injection of a phospholipid stock solution in methanol or as a suspension in assay buffer that had been subjected to probe sonication. This solution was excited at 350 nm and the fluorescence was measured at 500 nm with a Hitachi F2000 fluorimeter; data were recorded on a computer. Annexin V was added to the assay and left for 1 min before the addition of PLA₂ and the measurement of fluorescence displacement. Enzyme rates in the presence of annexin V were compared with rates from the standard assay containing enzyme only. Calibration of fluorescence displacement was performed with 0.2 mM arachidonic acid (SAPC, SAPA) or 0.2 mM oleic acid (DOPG).

RESULTS

Annexin V inhibits the cPLA₂-catalysed hydrolysis of DOPG but not SAPC

The kinetics of SAPC hydrolysis by cPLA₂ is unusual in that when using SUVs prepared by liposome sonication or by solvent injection a burst phase of hydrolysis is observed that rapidly

Figure 1 Effect of annexin V on SAPC and DOPG hydrolysis by cPLA₂

The inhibition of cPLA₂ by annexin V was determined with SAPC or DOPG (2.5 nmol/ml) as substrate in the form of SUVs, and 2.5 mM Ca²⁺. PLA₂ activity in the presence of annexin V was determined and is expressed as a percentage of the activity in the absence of annexin V. Assays were performed with 1 μ g of enzyme. Symbols: ■, DOPG; ▲, SAPC. Values are means $\pm$ S.D., $n = 3$.

decreases to a slow second phase. This burst phase seems to result from enzyme molecules being trapped on vesicles during hydrolysis [14,18]. We have argued that this trapping of enzyme molecules on the vesicles is of a co-operative nature and that the burst of hydrolysis reflects the hydrolysis of the outer surface of these vesicles [14]. It would be expected that the hydrolysis of SAPC by cPLA₂ would be only minimally inhibited in the presence of annexin V by a mechanism involving interfacial competition and sequestration because of the low affinity of annexin V for phosphatidylcholine interfaces.

The results of performing such an assay in the presence of 2.5 mM free Ca²⁺ are shown in Figure 1 and clearly demonstrate the inability of annexin V to inhibit cPLA₂ under the conditions used here. However, we have observed that cPLA₂ is also able to hydrolyse the anionic phospholipid DOPG at a significant rate, being approx. 15% of that seen with SAPC under these assay conditions. It would be expected that the hydrolysis of such an anionic substrate would be inhibited by annexin V: this is clearly seen in Figure 1. This inhibition, which is achieved with a limited substrate concentration (2.5 nmol) at 2.5 mM Ca²⁺, is half-maximal at approx. 1 μ g (28 pmol) of annexin V. Moreover, the stoichiometry of the inhibition curve suggests a substrate depletion mechanism involving a coating of the phospholipid vesicle surface by annexin V. Thus it would be expected that one annexin V molecule would cover approx. 30–60 molecules of phospholipid [7,19–21] and hence 2.5 nmol of phospholipid (which should equate to at least 1.25 nmol of the phospholipid in the outer monolayer of the SUV) would require between 42 and 84 pmol (1.5–3 μ g) of annexin V. The results shown in Figure 1 are clearly consistent with this stoichiometric relationship. The lack of inhibition seen when identical assays were performed with excess substrate (50 μ g/ml) is consistent with this mechanism of substrate depletion (results not shown).

Dependence of the ability of annexin V to inhibit cPLA₂ activity on Ca²⁺ concentration

In view of the low micromolar levels of Ca²⁺ found within the cell, it was important to study the relationship between Ca²⁺ and the effect of annexin V on cPLA₂. Rates of hydrolysis of SAPC vesicles increased significantly when Ca²⁺ levels were increased from micromolar to millimolar levels (Figure 2, upper panel), a result in line with assays reported previously with this substrate with the use of radiometric assays [22]. In contrast, the activity of cPLA₂ on SAPC SUVs was not affected to any significant extent by annexin V (10 μ g) over the range of Ca²⁺ concentrations used (Figure 2, upper panel). Once again this is expected because of the inability of the annexin to bind to a zwitterionic phospholipid.

The ability of annexin V to inhibit cPLA₂ activity and the requirement for Ca²⁺ is demonstrated in Figure 2 (lower panel), with DOPG as the phospholipid substrate. Annexin V (2 μ g) completely inhibited cPLA₂ activity at 2.5 mM Ca²⁺ but this effect was diminished as free Ca²⁺ levels were lowered; at 100 μ M Ca²⁺, 2 μ g of annexin V had no inhibitory action on the enzyme. This result must reflect the dependence of annexin V on Ca²⁺ concentration for binding to DOPG and thus its ability to sequester phospholipid substrate effectively from the enzyme.

Hydrolysis of SAPA by cPLA₂ is inhibited by annexin V in the presence of micromolar levels of Ca²⁺

Although we have been able to demonstrate, by using DOPG, the principle of cPLA₂ inhibition by substrate depletion, this is not of obvious physiological relevance, because phosphatidylglycerol is not a widely distributed phospholipid in eukaryotic

Figure 2 Effect of Ca²⁺ on the hydrolysis of SAPC and DOPG by cPLA₂ and inhibition by annexin V

All assays were performed with 2.5 nmol/ml phospholipid in the form of SUVs, and a range of Ca²⁺ concentrations was obtained with CaCl₂ and EGTA. Specific activities (■) shown were determined in the absence of annexin V. Rates of phospholipid hydrolysis in the presence of annexin V are expressed as a percentage of the activity in the absence of annexin V (▲). Upper panel, SAPC; lower panel, DOPG. Values are means ± S.D., *n* = 3.

Figure 3 Effect of annexin V on the hydrolysis of SAPA by cPLA₂

Phospholipid was present in assays as SUVs at a concentration of 2.5 nmol/ml. Symbols: ■, 20 μM Ca²⁺; ▲, 100 μM Ca²⁺. Enzyme activity on SAPA SUVs in the absence of annexin V is expressed as 100%. Values are means ± S.D., *n* = 3.

cells. However, SAPA can be generated within a biological membrane by the action of phospholipase D on the phosphatidylcholine substrate SAPC. As can be seen in Figure 3, annexin

V decreases the activity of cPLA₂ on SUVs composed of SAPA at 100 μM Ca²⁺. Again, this inhibition is Ca²⁺-dependent; at 20 μM Ca²⁺, up to 10 μg of annexin V has no significant effect on the enzyme. It should be noted that it was not possible to use Ca²⁺ concentrations above 100 μM with this phospholipid owing to a rapid decline in enzyme activity in this assay, presumably owing to vesicle aggregation.

Phosphatidic acid is one of several anionic phospholipids that have been reported to activate the hydrolysis of radioactive SAPC by cPLA₂; this activation was most marked by PtdIns(4,5)P₂ [23]. We observe a similar activation of SAPC hydrolysis by 2% (mol/mol) PtdIns(4,5)P₂ but such enhanced hydrolysis was not inhibited by annexin V even in the presence of 2.5 mM Ca²⁺ (results not shown).

DISCUSSION

Since the original discovery of annexin 1 (lipocortin 1) [1,2] as a potential mediator of the inflammatory effects of glucocorticoids, there has been considerable interest in Ca²⁺ and phospholipid-binding proteins as inhibitors of PLA₂. The early concept that these proteins caused inhibition by a direct interaction with PLA₂ (secretory PLA₂ at that time) has been replaced to a large extent by a substrate depletion model in which inhibition could be reversed by the presence of excess substrate. Although such a model might lack the immediate attraction of a mechanism involving the direct inhibition of the enzyme by annexin, the high concentration of annexin V in cells [10], coupled with its capacity to aggregate on low curvature surfaces [7,24], still offers a potentially effective mechanism for PLA₂ modulation within the cell by denying the enzyme access to crucial membrane domains.

In the present study we have taken advantage of a fluorescence displacement assay that allows the hydrolysis of low concentrations of a variety of phospholipid vesicles by PLA₂ to be monitored in terms of total fatty acids released. This method allows sufficient annexin V to be added to coat the interfacial surface completely and enabled the assay of human cPLA₂ activity with a variety of phospholipid substrates, some of which are not available as radioactive substrates.

The results have clearly established that under the assay conditions used *in vitro*, annexin V was unable to inhibit the hydrolysis of SAPC over a range of Ca²⁺ concentrations (1 μM–2.5 mM) as would be anticipated from the known inability of annexin V to bind to zwitterionic interfaces. The fact that no inhibition could be seen with annexin V at low concentrations of Ca²⁺ is of interest because Kim et al. [25] showed cPLA₂ inhibition by annexin I at low Ca²⁺ concentrations, which they proposed was by a specific interaction between annexin I and cPLA₂.

A mechanism involving specific interaction between the annexin and cPLA₂ finds indirect support from other reported properties of the annexins. For example, cPLA₂ has a CaLB domain, a feature in common with conventional forms of protein kinase C (PKC) and such PKCs are reported to be inhibited by annexin V [26]; however, the molecular mechanism of this inhibition of PKC remains obscure. It is of interest that recently a direct interaction between P120 GAP and annexin VI has been reported involving this CaLB domain [27]. The fact that cPLA₂ also has a CaLB domain provides a possible mechanism for direct modulation of this PLA₂ by annexins.

In contrast with negative results with SAPC, when an anionic substrate (DOPG) was used, prior addition of annexin V resulted in inhibition, whereas the stoichiometry of this inhibition was consistent with the normal substrate depletion mechanism. No inhibition was seen when the assays were performed in the presence of excess DOPG relative to added annexin V. The effect

of Ca^{2+} concentration on the inhibitory effect of annexin V was of particular interest. The results clearly established that under these assay conditions high cPLA₂ activity could be sustained by micromolar concentrations of Ca^{2+} , whereas annexin V inhibition by substrate depletion required Ca^{2+} concentrations in excess of 100 μM . A similar phenomenon was seen when SAPA, which could be generated from SAPC in the cell by the action of phospholipase D, was used as substrate. Again, cPLA₂ activity was inhibited by annexin V, but now at 100 μM Ca^{2+} , whereas no significant inhibition was seen at 20 μM Ca^{2+} .

Recent work demonstrates that, on activation, cPLA₂ translocates to the nuclear envelope, where binding to specific regions must occur [11,12]. It is of interest that it has now been shown that annexin V also translocates to this nuclear envelope on cell activation [13] and hence a modulatory role of this annexin on cPLA₂ cannot be discounted. At present the precise molecular species of phospholipid in the nuclear envelope that is hydrolysed by cPLA₂ is not known [28]. The identification of this species and the possible modulation of its availability for hydrolysis by annexin V is of obvious importance, particularly as part of the inflammatory response of appropriate cells. However, if annexin V were to have a role in modulating cPLA₂ activity, the results described would seem to rule out a mechanism simply involving substrate sequestration.

A modulatory role for annexin V in the cell could, however, involve an interaction with a specific membrane-associated protein in addition to binding to the phospholipid interface. Such proteins have already been proposed to explain the interaction of annexin with cell membranes at low Ca^{2+} concentrations [29] and might provide an explanation for the ability of annexin V to inhibit cPLA₂ activity in permeabilized HL-60 cells [30].

In summary, the ability of annexin V to inhibit cPLA₂ can be clearly demonstrated *in vitro* when using anionic phospholipid substrates in a mechanism involving substrate sequestration. However, the Ca^{2+} sensitivity of this process seems to rule out the possibility of such a simple mechanism operating *in vivo* despite the high concentrations of annexin V found in some tissues.

We thank Lawrence Hunt (BBSRC Centre for Molecular Recognition, Southampton, Hants., U.K.) for protein sequencing, and the BBSRC for a research studentship to A.G.B.

REFERENCES

- 1 Flower, R. J. and Blackwell, G. J. (1979) *Nature* (London) **278**, 456–459
- 2 Hirata, F., Schiffmann, E., Venkatasubramanian, K., Solomon, D. and Axelrod, J. (1980) *Proc. Natl. Acad. Sci. U.S.A.* **77**, 2533–2536
- 3 Moss, S. E. (ed.) (1992) in *The Annexins*, pp. 1–10, Portland Press, London
- 4 Clark, J. D., Schievella, A. R., Nalefski, E. A. and Lin, L. L. (1995) *J. Lipid Mediat. Cell Signall.* **12**, 83–117
- 5 Leslie, C. C. (1997) *J. Biol. Chem.* **272**, 16709–16712
- 6 Swairjo, M. A., Concha, N. O., Kaetzel, M. A., Dedman, J. R. and Seaton, B. A. (1995) *Nature Struct. Biol.* **2**, 968–974
- 7 Andree, H. A., Reutelingsperger, C. P., Hauptmann, R., Hemker, H. C., Hermens, W. T. and Willems, G. M. (1990) *J. Biol. Chem.* **265**, 4923–4928
- 8 Meers, P. and Mealy, T. (1993) *Biochemistry* **32**, 11711–11721
- 9 Meers, P. and Mealy, T. (1993) *Biochemistry* **32**, 5411–5418
- 10 Schlaepfer, D. D. and Haigler, H. T. (1990) *J. Cell Biol.* **111**, 229–238
- 11 Glover, S., Decarvalho, M. S., Bayburt, T., Jonas, M., Chi, E., Leslie, C. C. and Gelb, M. H. (1995) *J. Biol. Chem.* **270**, 15359–15367
- 12 Schievella, A. R., Regier, M. K., Smith, W. L. and Lin, L. L. (1995) *J. Biol. Chem.* **270**, 30749–30754
- 13 Raynal, P., Kuijpers, G., Rojas, E. and Pollard, H. B. (1996) *FEBS Lett.* **392**, 263–268
- 14 Creaney, A., Masters, D. J., Needham, M. B., Gordon, R. D., Mott, R. and Wilton, D. C. (1995) *Biochem. J.* **306**, 857–864
- 15 Goossens, E. L. J., Reutelingsperger, C. P. M., Jongasma, F. H. M., Kraayenhof, R. and Hermens, W. T. (1995) *FEBS Lett.* **359**, 155–158
- 16 Worrall, A. F., Evans, C. and Wilton, D. C. (1991) *Biochem. J.* **278**, 365–368
- 17 Wilton, D. C. (1990) *Biochem. J.* **266**, 435–439
- 18 Ghomashchi, F., Schuttel, S., Jain, M. K. and Gelb, M. H. (1992) *Biochemistry* **31**, 3814–3824
- 19 Huber, R., Berendes, R., Burger, A., Schneider, M., Karshikov, A., Luecke, H., Romisch, J. and Paques, E. (1992) *J. Mol. Biol.* **223**, 683–704
- 20 Meers, P., Daleke, D., Hong, K. and Papahadjopoulos, D. (1991) *Biochemistry* **30**, 2903–2908
- 21 Ravanat, C., Torbet, J. and Freyssinet, J. M. (1992) *J. Mol. Biol.* **226**, 1271–1278
- 22 Marshall, L. A. and McCarte Roshak, A. (1992) *Biochem. Pharmacol.* **44**, 1849–1858
- 23 Leslie, C. C. and Channon, J. Y. (1990) *Biochim. Biophys. Acta* **1045**, 261–270
- 24 Pigault, C., Follenius Wund, A., Schmutz, M., Freyssinet, J. M. and Brisson, A. (1994) *J. Mol. Biol.* **236**, 199–208
- 25 Kim, K. M., Kim, D. K., Park, Y. M., Kim, C. K. and Na, D. S. (1994) *FEBS Lett.* **343**, 251–255
- 26 Schlaepfer, D. D., Jones, J. and Haigler, H. T. (1992) *Biochemistry* **31**, 1886–1891
- 27 Davis, A. J., Butt, J. T., Walker, J. H., Moss, S. E. and Gawler, D. J. (1996) *J. Biol. Chem.* **271**, 24333–24336
- 28 Peters Golden, M., Song, K., Marshall, T. and Brook, T. (1996) *Biochem. J.* **318**, 797–803
- 29 Trotter, P. J., Orchard, M. A. and Walker, J. H. (1995) *Biochem. J.* **308**, 591–598
- 30 Mira, J. P., Dubois, T., Oudinet, J. P., Lukowski, S., Russo Marie, F. and Geny, B. (1997) *J. Biol. Chem.* **272**, 10474–10482