Volume 219, number 1, 176–180 FEB 04901 July 1987

Lipocortin inhibition of extracellular and intracellular phospholipases A_2 is substrate concentration dependent

A.J. Aarsman, G. Mynbeek, H. van den Bosch, B. Rothhut[+], B. Prieur[+], C. Comera[+], L. Jordan[+] and F. Russo-Marie[+]

Biochemistry Laboratory, Padualaan 8, 3584 CH Utrecht, The Netherlands and [+] *Unité des Venins, Unité Associée Institut Pasteur/INSERM U 285, 25 rue du Dr Roux, 75015 Paris, France*

Received 30 April 1987

Hydrolysis of *Escherichia coli* membrane phospholipids by pancreatic phospholipase A_2 was inhibited by lipocortin from human monocytes in a substrate dependent manner. Inhibition was completely overcome at substrate concentrations above 250 μM. Lipocortin also inhibited partially purified preparations of two intracellular phospholipases A_2 isolated from rat liver mitochondria and rat platelets when these enzymes were assayed at low micromolar concentrations of phosphatidylethanolamine. Inhibition gradually decreased with increasing substrate concentrations both for pancreatic and platelet phospholipase A_2 and became completely abolished above 15 and 50 μM phosphatidylethanolamine, respectively.

Lipocortin; Phospholipase A_2; Phosphatidylethanolamine

1. INTRODUCTION

Lipocortins belong to a family of proteins described initially as induced by glucocorticosteroids and able to inhibit phospholipase A_2 (see [1–4]). Since their initial description, they have been cloned and sequenced [5–7] and have been related to a family of calcium and phospholipid binding proteins, namely calpactins and calelectrins. Indeed, lipocortin I is identical to calpactin II, and lipocortin II identical to the heavy chain of calpactin I, both membrane cytoskeletal proteins, able to bind calcium, phospholipid and actin [8–10]. Calelectrin was initially described in the electric organ of *Torpedo marmorata* and later in mammalian tissues, as a protein able to bind calcium and chromaffin granule membranes, and was considered as a candidate for Ca^{2+} dependent regulators of membrane events [11–13]. The lipocortins, the calpactins and the calelectrins all possess a 17 amino acid residue consensus sequence [14–16].

The way lipocortins and related proteins inhibit phospholipase A_2 is still a matter of debate. Initially, a direct inhibition by protein-protein interaction was implicated [17] although inhibition of bacterial phospholipase C and plant phospholipase D in vitro by lipomodulin, a former name for lipocortin [17], challenged such a specific protein-phospholipase A_2 interaction. The recent recognition that lipocortins are able to bind to phospholipids, the substrates for phospholipase A_2, suggested possibilities for alternative modes of actions. In addition, phospholipase A_2 inhibition by lipocortins from various sources has generally been assayed by using porcine pancreatic phospholipase A_2 and either highly labeled *Escherichia coli* membranes [5–7,18] or phosphatidylcholines [17,19], both at very low substrate concentrations in the micromolar range. Indeed a recent paper by Davidson et al. [20]

Correspondence address: H. van den Bosch, Biochemistry Laboratory, Padualaan 8, 3584 CH Utrecht, The Netherlands

Published by Elsevier Science Publishers B.V. (Biomedical Division)

demonstrated that phospholipase A_2 inhibition by calpactins isolated from bovine lung was substrate dependent. While 100% inhibition was observed at 2 μM *E. coli*-derived phospholipid vesicles, no inhibition was found with this substrate above 8 μM. However, inhibition was only studied for the extracellular phospholipase A_2 from porcine pancreas. In this paper, we studied the effect of human lipocortins on this enzyme as well as on two partially purified preparations of intracellular phospholipases A_2 obtained from rat liver mitochondria and rat platelets.

2. MATERIALS AND METHODS

[^{3}H]Oleic acid (4.2 Ci/mmol) was purchased from Amersham, Paris; fatty acid free bovine serum albumin (BSA) was from Sigma, St. Louis, MO; and porcine pancreatic phospholipase A_2 (700 Units/mg) from Boehringer, Mannheim.

2.1. *Isolation of proteins*

2.1.1. Lipocortin

Lipocortin (32 kDa) was isolated from peripheral mononuclear cells as described in the accompanying paper [27]. The proteins were characterized by their migration in gel electrophoresis, by their isoelectric point and by Western blotting using a monoclonal antibody raised against rat renal lipocortin and a polyclonal anti-32-kDa antibody.

2.1.2. Platelet phospholipase A_2

Rat platelet phospholipase A_2 was partially purified from platelet extracts prepared as described [21]. Briefly, platelet lysates were extracted for 1 h at 4°C with a buffer containing 25 mM Tris-HCl (pH 7.4) and 1 M KCl. After centrifugation for 1 h at 100000 × *g*, the clear supernatant containing over 85% of the platelet phospholipase A_2 activity, was chromatographed over a Sepharose 4B column to which a monoclonal antibody against rat liver mitochondrial phospholipase A_2 had been coupled. As shown [22] this monoclonal antibody cross-reacted with rat platelet phospholipase A_2. After more than 95% of the applied proteins had been eluted with the application buffer, phospholipase A_2 activity was eluted with a glycine buffer (pH 2.5) in yields of 150–200%. The high yields are probably due to the removal of inhibiting compounds present in the platelet extract [22]. Details of this purification will be published elsewhere. The amount of protein in the final preparation was too low to allow for an accurate determination.

2.1.3. Liver mitochondrial phospholipase A_2

Rat liver mitochondrial phospholipase A_2 was purified as described [22] by AcA 54 chromatography of delipidated mitochondrial extracts. The specific activity of this preparation amounted to 350 nmol · min^{-1} · mg protein^{-1}.

2.2. *Assay of porcine pancreatic phospholipase A_2 using* E. coli *membranes*

The phospholipase A_2 inhibitory assay was performed using [^{3}H]oleic acid-labeled *E. coli* membranes as described in [18]. The proteins were preincubated for 10 min with 100 ng of porcine pancreatic phospholipase A_2 in a final volume of 350 μl with 100 mM Tris-HCl, pH 8.0, 10 mM Ca^{2+}, at 4°C. Autoclaved [^{3}H]oleic acid-labeled, whole *E. coli* membranes were added as substrate to initiate the reaction which was terminated 10 min later by adding 100 μl of 2N HCl. 100 μl of fatty acid free BSA (100 mg/ml) was then added, the tubes were vortexed and centrifuged 5 min at 10000 × *g*. 200 μl of the supernatant containing released [^{3}H]oleic acid was counted by liquid scintillation spectrometry. Results were expressed as percentage inhibition of released oleic acid in the presence or absence of the proteins to be tested minus blanks where phospholipase A_2 and the inhibitory proteins were omitted.

2.3. *Assay of antiphospholipase A_2 activity using phosphatidylethanolamine substrate*

Phospholipases were preincubated for 10 min with the indicated amount of lipocortin and 10 mM Ca^{2+} in 100 mM Tris-HCl, pH 8.5, at 4°C prior to addition of 1-acyl-2-[1-^{14}C]linoleoyl-phosphatidylethanolamine. Substrate concentrations and specific radioactivities were variable as specified in the figure legends. After 30 min incubation at 37°C, released [^{14}C]linoleate was determined by a modified Dole extraction procedure as described in [21]. All experiments, including blanks containing neither enzymes nor lipocortin, were carried out in duplicate.

2.4. *Phospholipid measurement*

Phospholipids were extracted according to the procedure described by Bligh and Dyer [23]. After evaporation under nitrogen, the phosphate content was measured according to Böttcher et al. [24].

2.5. *Protein measurement*

Proteins were measured as described by Lowry et al. [25].

3. RESULTS AND DISCUSSION

Fig.1 shows the effect of increasing concentrations of [^{3}H]oleate-labeled *E. coli* membranes on the inhibition of pancreatic phospholipase A_2 by lipocortin from human monocytes. At a membrane concentration equivalent to 15 μM phospholipid the enzyme was inhibited for 72% by 2.5 μg lipocortin. This inhibition gradually decreased with increasing substrate concentration until at 250 μM substrate, the inhibition was completely abolished. These results are in good agreement with the recent data of Davidson et al. [20]. When using the same enzyme and phospholipid substrate in the form of whole *E. coli* cells these authors noted that inhibition by bovine lung calpactins ceased above 200 μM phospholipid. To see whether or not this phenomenon was specific for the extracellular pancreatic phospholipase A_2 in combination with the negatively charged *E. coli* phospholipids, we investigated the effect of lipocortin on two intracellular phospholipases A_2 using the neutral phosphatidylethanolamine as substrate.

Initial experiments in this series indicated that at a substrate concentration of 200 μM 1-acyl-2-[1-^{14}C]linoleoylphosphatidylethanolamine, neither pancreatic phospholipase A_2 (4 ng) nor rat liver mitochondrial phospholipase A_2 (20 ng, based on a spec. act. of 8 Units/mg [26]) were inhibited by the 32-kDa lipocortin up to amounts of 7.5 μg. However, when the substrate concentration was reduced to 2 μM, the same amounts of pancreatic and mitochondrial enzyme were inhibited 80% and 42%, respectively, by 7.5 μg lipocortin. Under similar conditions, an amount of rat platelet phospholipase A_2 releasing 0.43 nmol of linoleate was inhibited 56%. The inhibition of rat platelet phospholipase A_2 at low substrate concentrations is dose-dependent (fig.2). This inhibition reaches a plateau at 75% for 3 μg of the 32-kDa lipocortin. This could indicate that either the enzyme-lipocortin complex has some residual activity or that the phosphatidylethanolamine-lipocortin

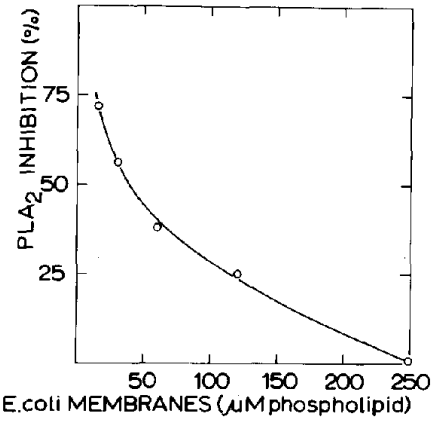

Fig.1. Effect of *E. coli* membrane substrate concentration on inhibition of pancreatic phospholipase A_2 by lipocortin. The incubations contained 0.1 μg phospholipase A_2 and variable amounts of [^{3}H]oleate-labeled *E. coli* membranes equivalent with the indicated concentrations of phospholipids in the absence or presence of 2.5 μg of the 32-kDa lipocortin from human monocytes.

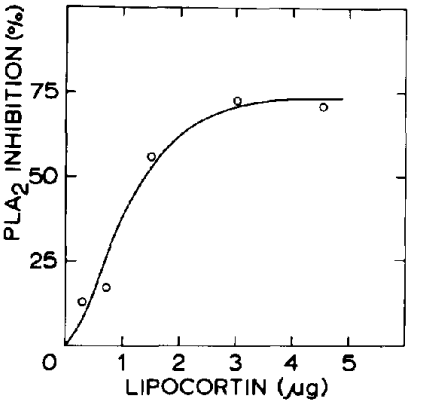

Fig.2. Dose response of lipocortin on rat platelet phospholipase A_2 inhibition. Incubations contained 5.0 μM 1-acyl-2-[1-^{14}C]linoleoyllphosphatidylethanolamine (spec. act. 4000 dpm/nmol) and a constant amount of platelet phospholipase A_2 in the absence or presence of the indicated amounts of lipocortin. In the absence of lipocortin, the enzyme released 0.36 nmol linoleate.

complex is still a substrate for the platelet phospholipase A_2, though inferior compared to phosphatidylethanolamine alone. In the former case, if lipocortin interacted solely with the enzyme, one would expect the lipocortin inhibition of the phospholipase A_2 to be independent of the substrate concentration. On the other hand, if lipocortin exerted its effects by binding to substrate or if the enzyme-lipocortin complex dissociated in the presence of phospholipid and Ca^{2+}, one would expect the phospholipase A_2 inhibition to decrease with increasing substrate concentration. In agreement with fig.1, the latter phenomenon is also seen with phosphatidylethanolamine as substrate, both for rat platelet and porcine pancreatic phospholipase A_2 (fig.3). However lipocortin inhibition of platelet phospholipase A_2 appears somewhat less sensitive to increasing phosphatidylethanolamine concentrations than observed for pancreatic phospholipase A_2.

In summary, the data have shown that inhibition of the extracellular pancreatic phospholipase A_2 by human monocyte lipocortin is dependent on the amount of *E. coli* membrane substrate in a manner corroborating recently published experiments using a similar assay system and bovine lung calpactins. In addition, we have shown that two

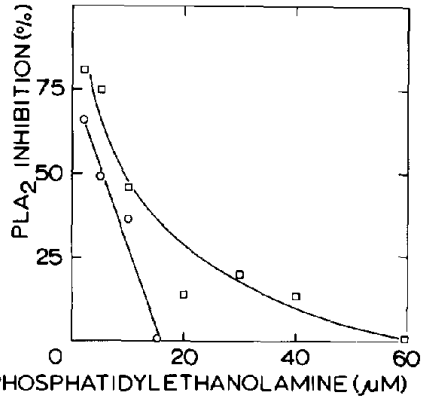

Fig.3. Effect of phosphatidylethanolamine concentration on phospholipase A_2 inhibition by lipocortin. Substrate (as in fig.2) concentration was varied as indicated both in the absence or presence of 3 μg lipocortin. (○—○) Pancreatic phospholipase A_2 (4 ng); (□—□) an amount of rat platelet phospholipase A_2 releasing 0.16 nmol and 1.5 nmol of linoleate at 2 μM and 40 μM substrate, respectively.

phospholipases A_2 from intracellular origin, i.e. rat liver mitochondria and rat platelets are likewise inhibited by lipocortin at low concentrations of a neutral phospholipid substrate. However, also in these cases, lipocortin inhibition of phospholipase A_2 activity is completely abolished at substrate concentrations above 50 μM. At present it cannot be decided whether this relief of inhibition is caused by direct lipocortin-phospholipid interactions that have been described recently [8–10] or to dissociation of inactive phospholipase A_2-lipocortin complexes in the presence of phospholipids. Whatever the detailed molecular mechanism, it is clear, however, that these results have important implications for the proposed functions of lipocortins as regulators of intracellular phospholipase A_2 activity in response to glucocorticosteroid treatment of cells.

REFERENCES

[1] Flower, R.J., Wood, J.N. and Parente, L. (1984) Adv. Inflamm. Res. 7, 61–69.
[2] Hirata, F., Schiffmann, D., Venkatasubramanian, K., Salomon, D. and Axelrod, J. (1981) Proc. Natl. Acad. Sci. USA 77, 2533–2536.
[3] Rothhut, B. and Russo-Marie, F. (1986) Adv. Inflamm. Res. 10, 360–363.
[4] DiRosa, M., Flower, R.J., Hirata, F., Parente, L. and Russo-Marie, F. (1984) Prostaglandins 28, 441–444.
[5] Pepinsky, R.B., Sinclair, L.K., Browning, J.L., Mattaliano, R.J., Smart, J.E., Chow, E.P., Falbel, T., Ribolini, A., Garwin, J. and Wallner, B.P. (1986) J. Biol. Chem. 261, 4246–4249.
[6] Wallner, B.P., Mattaliano, R.J., Hession, C., Cate, R.L., Tizard, R., Sinclair, L.K., Foeller, C., Chow, E.P., Browning, J.L., Ramachandran, K.L. and Pepinsky, R.B. (1986) Nature 320, 77–80.
[7] Huang, K.-S., Wallner, B.P., Mattaliano, R.J., Tizard, R., Burne, C., Frey, A., Hession, C., McGray, P., Sinclair, L.K., Chow, E.P., Browning, J.L., Ramachandran, K.L., Tang, J., Smart, J.E. and Pepinsky, R.B. (1986) Cell 46, 191–199.
[8] Glenney, J.R. (1986) Proc. Natl. Acad. Sci. USA 83, 4258–4262.
[9] Kristensen, T., Saris, C.J.M., Hunter, T., Hicks, L.J., Noonan, D.J., Glenney, J.R. and Tack, B. (1986) Biochemistry 25, 4497–4503.
[10] Glenney, J.R. (1986) J. Biol. Chem. 261, 7247–7252.

[11] Walker, J.H. (1982) J. Neurochem. 39, 815–823.
[12] Südhof, T.C., Ebbecke, M., Walker, J.H., Fritsche, U. and Boustead, C. (1984) Biochemistry 23, 1103–1109.
[13] Geisow, M.J., Fritsche, U., Hexham, J.M., Dash, B. and Johnson, T. (1986) Nature 320, 636–638.
[14] Geisow, M.J. (1986) FEBS Lett. 203, 99–103.
[15] De, B.K., Misono, K.S., Kukas, T.J., Mroczkowski, B. and Cohen, S. (1986) J. Biol. Chem. 261, 13784–13792.
[16] Saris, C.M.J., Tack, B.F., Kristensen, T., Glenney, J.R. and Hunter, T. (1986) Cell 46, 201–212.
[17] Hirata, F. (1981) J. Biol. Chem. 256, 7730–7733.
[18] Rothhut, B., Russo-Marie, F., Wood, J., DiRosa, M. and Flower, R.J. (1983) Biochem. Biophys. Res. Commun. 117, 878–884.
[19] Hirata, F., Notsu, Y., Iwata, M., Parente, L., DiRosa, M. and Flower, R.J. (1982) Biochem. Biophys. Res. Commun. 109, 223–230.
[20] Davidson, F.F., Dennis, E.A., Powell, M. and Glenney, J.R. jr (1987) J. Biol. Chem. 262, 1698–1705.
[21] Aarsman, A.J., Roosenboom, C.F.P., Van Geffen, G.E.W. and Van den Bosch, H. (1985) Biochim. Biophys. Acta 837, 288–295.
[22] De Jong, J.G.N., Amesz, H., Aarsman, A.J., Lenting, H.B.M. and Van den Bosch, H. (1987) Eur. J. Biochem. 167, 129–135.
[23] Bligh, E.G. and Dyer, W.J. (1959) Can. J. Biochem. Physiol. 37, 911–917.
[24] Böttcher, C.F.J., Van Gent, C.M. and Pries, C. (1961) Anal. Chim. Acta 24, 203–204.
[25] Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275.
[26] De Winter, J.M., Vianen, G.M. and Van den Bosch, H. (1982) Biochim. Biophys. Acta 712, 332–341.
[27] Rothhut, B., Comera, C., Prieur, B., Errasfa, M., Minassian, G. and Russo-Marie, F. (1987) FEBS Lett. 219, 169–175.