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Lipocortin I and lipocortin II inhibit phosphoinositide- and polyphosphoinositide-specific phospholipase C

The effect results from interaction with the substrates

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Lipocortins I and II, known to inhibit phospholipase A_2, have been purified from bovine lung and tested with respect to their ability to affect the enzymatic activities of phosphoinositide- and polyphosphoinositide-specific phospholipase C from human platelets, rat liver cytosol or rat brain membranes. At 0.67 μM, both lipocortins led to complete inhibition of phospholipase C activity with either phosphatidylinositol or phosphatidylinositol 4,5-bisphophate as substrate. The inhibition could be overcome by increasing the substrate concentration. Ultracentrifugation studies with lipocortin II showed a direct interaction between phosphatidylinositol and the lipocortin, indicating that the lipocortins inhibit phospholipase C not directly but by interacting with the substrate. In experiments with plasma membranes from [^{3}H]inositol-labeled HL-60 cells, lipocortin II did not affect PI-specific phospholipase C activity in the absence or presence of calcium plus or minus GTP-γ-S.

Calpactin; Lipocortin; Phospholipase C, polyphosphoinositide-specific

1. INTRODUCTION

The lipocortins represent a group of calcium- and phospholipid-binding proteins which have been implicated in the control of phospholipase A_2 [1–3]. The lipocortins belong to the calpactin family which is represented mainly by four proteins: (i) calpactin I (= lipocortin II), a 36 kDa protein serving as substrate for the pp60^{src} kinase and protein kinase C [4–8]; (ii) calpactin II (= lipocortin I), a 35 kDa protein serving as substrate for the EGF-receptor tyrosine kinase and protein kinase C [8–12]; (iii) protein I which represents a tetrameric protein consisting of two p36 protomers and two 11 kDa proteins related to the S-100 protein [13]; and (iv) p32, a 32 kDa protein which can serve as substrate for protein kinase C [6,14]. An inhibitory effect of lipocortin I was first described by Hirata et al. [1,2]. Davidson et al. [15] later showed that lipocortin II also inhibited this phospholipase. More recently, Glenney's group [16–18] reported that the inhibitory effect of lipocortin II results from binding of the lipocortin to the phospholipid substrate rather than from a direct effect on phospholipase A_2 itself.

Here, we have addressed the question as to whether lipocortins might affect the activity of phosphoinositide- and polyphosphoinositide-specific phospholipase(s) C. As these enzymes are directly involved in various signal transduction processes, their regulation by lipocortins could be of potential importance.

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2. MATERIALS AND METHODS

2.1. *Materials*

Phospholipase A_2 from pig pancreas was obtained from

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Sigma (Munich); DEAE-cellulose DE52 from Whatman (Springfield, UK); Sephadex G-100 and G-150, Sephacryl S-200-HR, heparin-Sepharose-CL-B, phenyl-Sepharose 4B-CL, Mono-Q anion exchanger and Mono-S cation exchanger from Pharmacia (Freiburg/Brg). All chemicals were analytical grade, obtained from E. Merck (Darmstadt). *myo*-[^{3}H]Inositol (spec. act. 110 Ci/mmol) and [9,10(n)-^{3}H]oleic acid (spec. act. 8 Ci/mmol) were supplied by Amersham-Buchler (Braunschweig). ^{3}H-labeled *E. coli* membranes which served as substrate for phospholipase A_2 were prepared from [^{3}H]oleic acid according to Davidson et al. [15]. [^{3}H]Inositol-labeled phosphatidylinositol was prepared by incubating 250 μCi carrier-free [^{3}H]inositol with 600 mg rat liver microsomal protein and purifying [^{3}H]phosphatidylinositol by thin-layer chromatography (TLC) as in [19]. Microsomes were prepared according to Takenawa and Egawa [20].

2.2. *Methods*

2.2.1. Preparation of membranes from undifferentiated HL-60 cells

About 200×10^6 undifferentiated cells were grown in Clicks RPMI medium without DMSO under 5% CO_2 at 37°C. Cells were transferred to medium 199 and incubated for 18 h in the presence of 1 mCi carrier-free [^{3}H]inositol. Cells were subsequently washed three times each with phosphate-buffered saline, washing buffer B (137 mM NaCl, 2.7 mM KCl, 20 mM Pipes, 5.6 mM glucose, 1 mg/ml of bovine serum albumin, 1 mM *myo*-inositol; pH 6.8) and washing buffer C (= washing buffer B without *myo*-inositol). Membrane preparation was performed according to a modification of the methods of Stutchfield and Cockroft [21] and Hrbolich et al. [22].

2.2.2. Measurement of polyphosphoinositide-specific phospholipase C activity in membranes from HL-60 cells

Incubation was carried out in the following medium (final concentrations): 25 mM Hepes, 100 mM KCl, 2.5 mM EGTA, 1.23 mM ATP, 1.6 mM $MgCl_2$, 10 mM LiCl; pH 7.4. The total volume was 220 μl. Reaction was initiated by simultaneous addition of labeled HL-60 membranes and calcium. The total final concentration of calcium was 2.5 mM, and the calculated free Ca^{2+} concentration amounted to 10^{-7} M. When GTP-γ-S was used, it was added to the medium prior to addition of membranes. After 10 min incubation at 37°C, the reaction was stopped by addition of 600 μl of 6% (w/v) trichloroacetic acid. Following centrifugation the supernatant was washed three times with diethyl ether and subsequently brought to pH 7.0 by titration with 1 M triethanolamine, pH 7.0. The sample was loaded onto a small (1.4 ml) column of Dowex 1×8 (200–400 mesh) and inositol and glycerophosphoinositol were eluted with 60 mM ammonium formate/5 mM NaB_4O_6. The total phosphoinositols were eluted with 1.2 M ammonium formate/0.1 M formic acid and 4-ml aliquots were mixed with 16 ml Unisolve 1, radioactivity being measured in a liquid scintillation spectrometer.

2.2.3. Preparation of lipocortins I and II

Fresh bovine lungs for the preparation of lipocortins were obtained from a local slaughterhouse. The first steps in this procedure including DEAE-cellulose chromatography were performed according to Khanna et al. [23]. Protein fractions eluting between 0 and 50 mM NaCl were concentrated by Amicon P-10 filtration in the presence of 20 mM Tris-Cl, 2 mM DTE, 1 mM EGTA and 150 mM NaCl, pH 7.4 (0°C) to 2 ml and applied to a Sephacryl-S 200-HR column. The first protein peak eluting from the column represented the pI protein, with the next one corresponding to mainly lipocortin I and II. Fractions of the second peak were concentrated in the presence of 50 mM Mes (pH 6.0) and applied to a Mono-S column. Lipocortin I was separated from lipocortin II by FPLC using a gradient from 0 to 300 mM NaCl. As lipocortin I and II did not clearly separate during SDS-PAGE, the identity of the peak containing lipocortin II was confirmed by the use of a monoclonal anti-lipocortin II antibody, and from the position of the 36 kDa protein which had been generated from the pI protein by treatment with 9 M urea and which eluted from the Mono-S column at the same ionic strength as lipocortin II.

2.2.4. Preparation of polyphosphoinositide-specific phospholipase(s) C

Phospholipase C from human platelets was purified according to Manne and Kung [24]. The enzyme from rat liver cytosol was purified according to Takenawa and Nagain [19]. The enzyme from rat brain cortex membranes was enriched as described by Litosch [25].

Determination of the activities of phospholipase A_2 and phospholipase C was performed as given in the legends to fig.1 and table 1, respectively.

2.2.5. Binding of phosphatidylinositol to lipocortin II

Binding was evaluated by discontinuous sucrose gradient centrifugation using a modification of the method of Davidson et al. [15], except that [^{3}H]phosphatidylinositol instead of radioactive phosphatidylserine was used. The final concentration of lipocortin (as calculated for the total volume of the gradient) was 0.5 μM, that of phosphatidylinositol (in the form of liposomes) being 65 μM. In contrast to Davidson et al. [15], we did not use phosphatidylethanolamine in addition to the labeled phospholipid, but employed only sonicated phosphatidylinositol. Samples (5 ml/tube) were spun on $300000 \times g_{av}$ in a Spinco SW-65 swing-out rotor for 2 h. Continuation of centrifugation for another 12 h did not significantly change the positions of the various compounds in the gradient. The tubes were punched from the bottom, 8 fractions collected from each tube, and the tubes analyzed for protein (by determining protein concentration and via SDS-PAGE) and phospholipid radioactivity.

3. RESULTS

3.1. *Inhibition of pancreatic phospholipase A_2*

In two experiments, we investigated the effect of 0.2 μM lipocortin I and II on pancreatic phospholipase A_2 activity in order to monitor the system. Under the experimental conditions used, lipocortin I resulted in 94 and 89% inhibition, with the values for lipocortin II being 90 and 89%.

In accordance with the findings of Davidson et al. [15], this inhibition could be suppressed by increasing substrate concentration (fig.1).

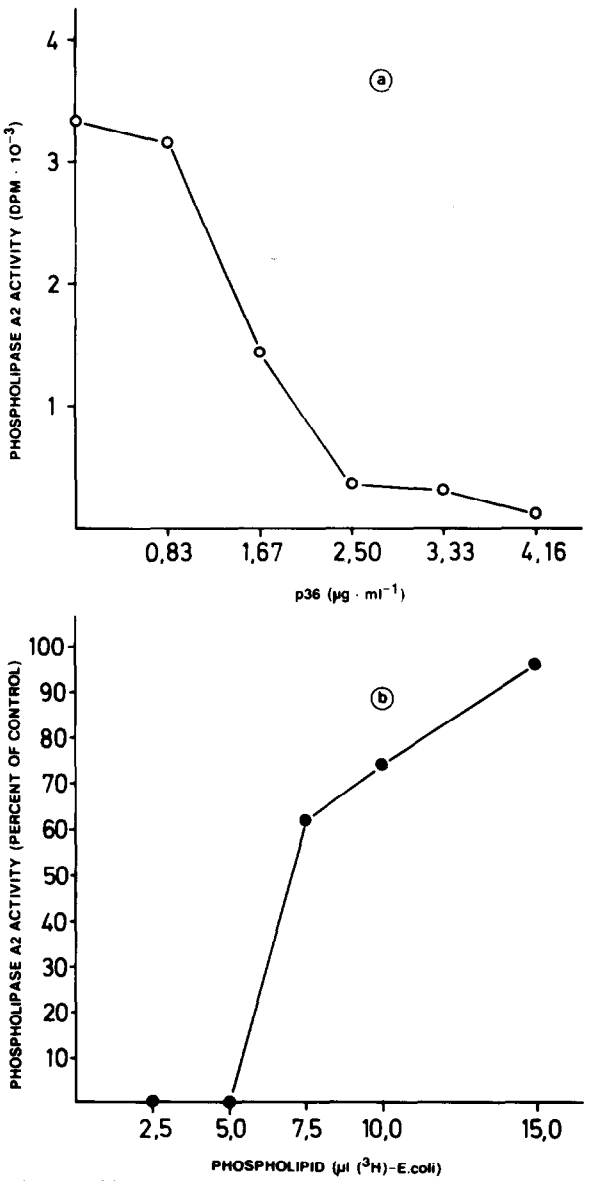

Fig.1. Inhibition of pig pancreatic phospholipase A_2 by lipocortin II (p36). The test mixture (0.4 ml) contained (final concentrations) 88 mM Tris-Cl, pH 7.9 (25°C), 9.6 mM $CaCl_2$, and 12.5 µl ^{3}H-labeled *E. coli* lipids which had been prepared according to [15]. The reaction was started by addition of 0.1 µg pig pancreatic phospholipase A_2. After 5 min at 25°C the reaction was stopped by addition of 150 µl of 2 M HCl followed by 200 µl of a 10% (w/v) solution of bovine serum albumin in water. The mixture was vortex-mixed, spun for 15 min at 15000 × *g*, and 400 µl of the supernatant was used for liquid scintillation counting. (a) Dependency of inhibition on the concentration of lipocortin II (p36). (b) The inhibition by p36 is overcome by increasing the substrate concentration. The reaction was performed as in (a) in the presence of 2.5 µg/ml (= 69 nM) p36. The amount of labeled *E. coli* lipids was increased from 2.5 to 15 µl. Total test volume was 0.4 ml.

3.2. *Effects on polyphosphoinositide-specific phospholipases C*

The effects of the two lipocortins observed with three different preparations of polyphosphoinositide-specific phospholipase C are presented in table 1. At 1.65 µM, both lipocortins completely abolished the cleavage of phosphatidylinositol. The same holds true for the cleavage of phosphatidylinositol 4,5-bisphosphate (fig.2b). In experiments with phosphatidylinositol as substrate, inhibition could be partially overcome by increasing the substrate concentration (fig.2a). Experiments with phosphatidylinositol 4,5-bisphosphate were carried out over a concentration range where the lipocortin effect could not be suppressed.

3.3. *Interaction between phosphatidylinositol and lipocortin II*

The results obtained on ultracentifugation show that lipocortin II banded in the most heavy fraction when spun alone (fig.3a), whereas, under identical conditions, the phosphatidylinositol liposomes appeared in fractions 4 and 5 (fig.3a).

Table 1

Inhibition of phosphoinositide-specific phospholipase C from different sources by lipocortin II (p36) and lipocortin I (p35)

Source of phospholipase C	[Inhibitor] (µmol/l)	Phospholipase C activity (dpm in Ins-P)		Inhibition (%)	
		Expt 1	Expt 2	Expt 1	Expt 2
Rat liver	–	5286	6192	0	0
	1.67 µM p36	72	132	99	98
Rat brain	–	4180	4240	0	0
	1.61 µM p36	0	0	100	100
	1.66 µM p35	0	36	100	99
Human platelets	–	5854	5320	0	0
	1.61 µM p36	0	0	100	100
	1.66 µM p35	56	0	99	100

Phospholipase activity was assayed over 20 min at 37°C in 50 mM Tris-maleate, pH 6.0, containing 2 mM $CaCl_2$ and 10 µM [^{3}H]phosphatidylinositol (spec. act. about 55000 dpm/nmol). The reaction was stopped by addition of 2 ml $CHCl_3$/CH_3OH/HCl (100:100:0.6, by vol.) and the phospholipids extracted following addition of 0.5 ml of 1 M KCl. After phase separation by centrifugation, 0.5 ml of the aqueous phase were removed for liquid scintillation counting of radioactive inositol 1-phosphate (Ins-P)

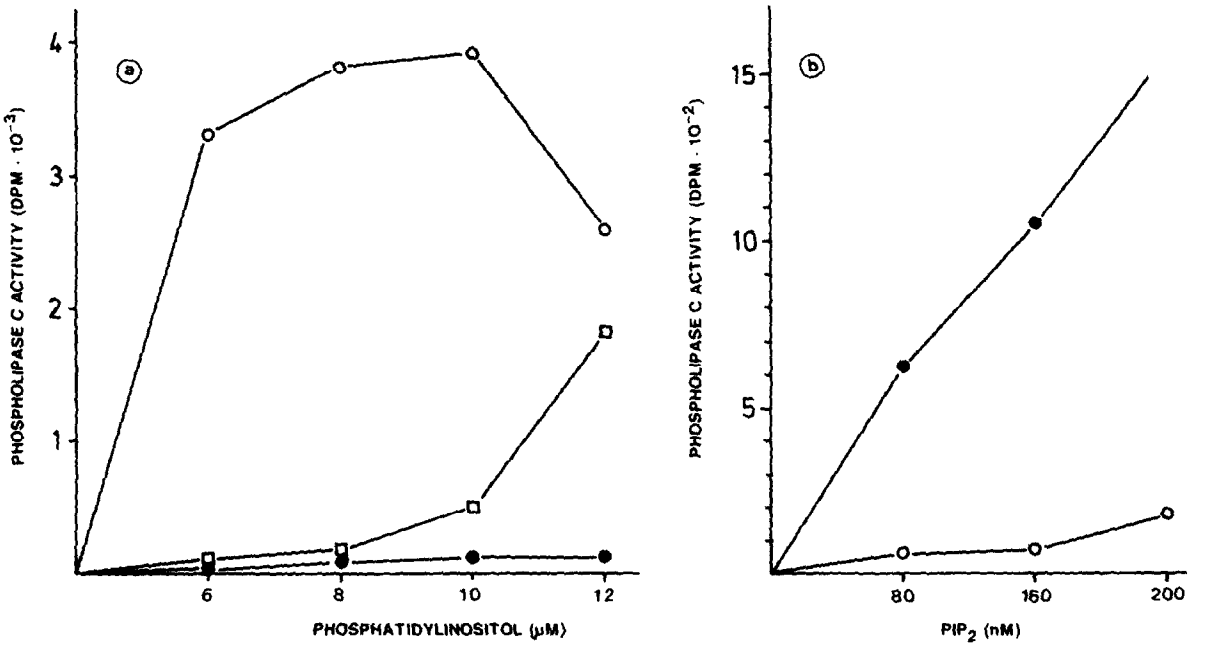

Fig.2. Inhibition of phosphoinositide-specific phospholipase C from human platelets (a) and rat brain cortex membranes (b) l 0.67 μM lipocortin II (p36). (a) Effect of increasing concentrations of phosphatidylinositol on reaction velocity in the presence ar absence of p36. (○—○) No p36; (●—●) controls without enzyme; (□—□) reaction in the presence of p36. Note that up to substrate concentration of 8 μM the reaction is almost completely inhibited, but that a further increase of phosphatidylinositol restor the reaction. (b) Substrate: phosphatidylinositol 4,5-bisphosphate (PIP_2). (●—●) Reaction in the absence of p36 and (○—○) in th presence of p36. Note that increasing the substrate concentration to 200 nM is insufficient for suppression of inhibition.

When added together at a free calcium concentration of 10 μM, the positions of both compounds changed and they appeared together in fraction 3 (fig.3b). On reduction of the free calcium concentration, only a small fraction of the radioactive phosphatidylinositol banded together with the lipocortin and the bulk of the protein was again recovered in the most dense fraction (fig.3c).

3.4. *Experiments with HL-60 cells*

Membranes prepared from undifferentiated HL-60 cells released a significant amount of labeled phosphoinositols in the presence of 100 nM free calcium. The addition of 50 μM GTP-γ-S doubled the amount of released phosphoinositols (table 2). The presence of 1.5 μM lipocortin II did not lead to inhibition of this effect.

4. DISCUSSION

According to the present results, the inhibitory action of lipocortins is not restricted to the phospholipase A_2 reaction alone, but also affects the cleavage of polyphosphoinositides by phospholipase(s) C. However, inhibition of the phospholipase C-catalyzed reaction seems to result from interaction between the lipocortins with the substrate phospholipid and not from direct action on the enzyme. This is further supported by our finding that the hydrolysis of phosphatidylcholine by phospholipase C from *Bacillus cereus* was not inhibited by lipocortin I or II (not shown). This is in line with data reported by Glenney's group, which show that the lipocortins interact with the acidic phospholipid phosphatidylserine, but not at all, or only very slightly with neutral phospholipids like phosphatidylcholine [15]. The experiments with membranes from HL-60 cells also show that lipocortin II was unable to inhibit the membrane-associated polyphosphoinositide-specific phospholipase C.

Considering the reports from Glenney's group and the data presented here, it seems highly unlikely that the main physiological function of lipocor-

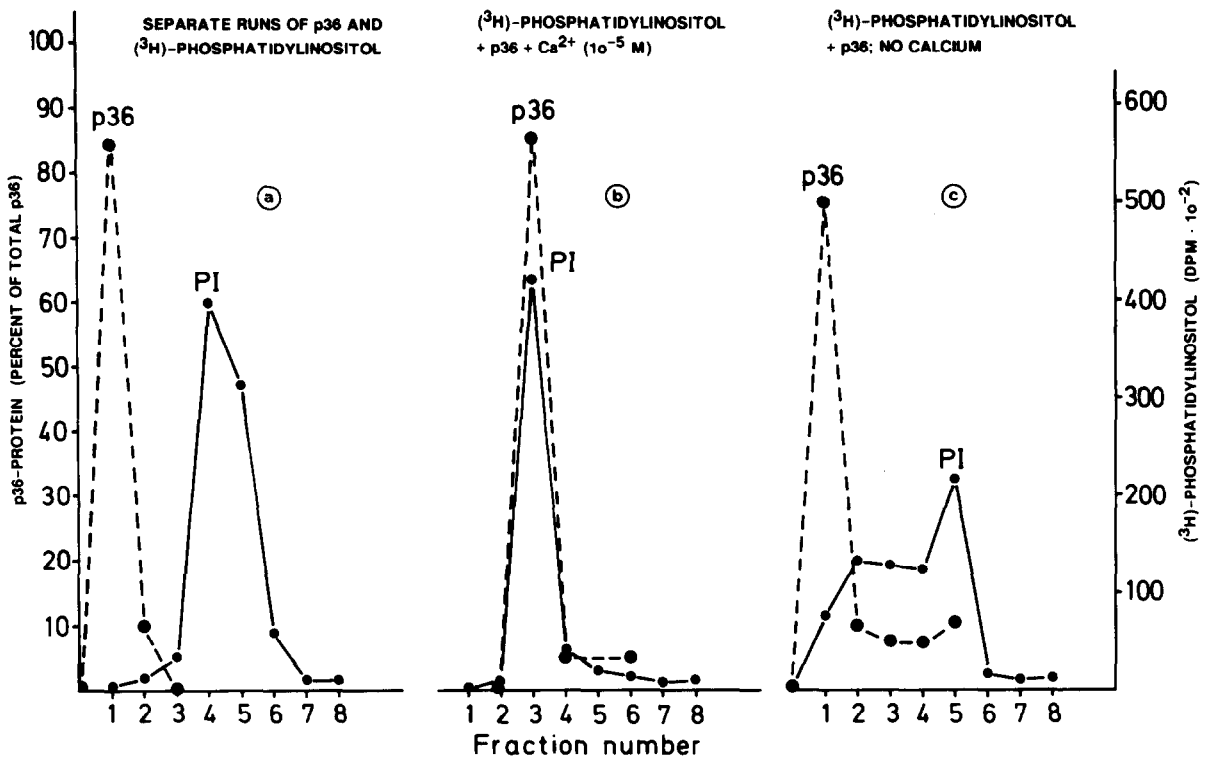

Fig.3. Calcium-dependent interaction between p36 and phosphatidylinositol during ultracentrifugation in a sucrose gradient. Experiments were carried out as given in section 2. In (a) p36 and [^{3}H]phosphatidylinositol (PI) were run separately. In (b) the two components were run together in the presence of 10 μM [Ca^{2+}]; in (c) the running conditions were as in (b) except that [Ca^{2+}] was lowered to $<10^{-8}$ M.

tins is the regulation of phospholipase activities. However, as members of the calpactin family share the property of interacting in a calcium-dependent manner with acidic phospholipids with such important proteins as protein kinase(s) C, the participation of the calpactins in signal-regulated control functions seems possible.

Table 2

GTP-γ-S stimulated hydrolysis of phosphoinositides in plasma membranes from [^{3}H]inositol-prelabeled undifferentiated HL-60 cells: lipocortin II (p36) has no inhibitory effect

Experimental conditions	Total inositol phosphates released (dpm)
Control cells	8380
GTP-γ-S (50 μM)	19105
plus p36 (1.5 μM)	20475

HL-60 cells were labeled and plasma membranes equivalent to 2.65×10^6 dpm were incubated in the presence of 10^{-7} M free calcium as given in section 2. Total inositol phosphates represent the sum of inositol 1-phosphate, inositol 1,4-bisphosphate, inositol 1,4,5-trisphosphate, inositol 1,3,4-trisphosphate and forms with a higher degree of phosphorylation

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