Calcium-dependent binding of uteroglobin (PCB-BP/CCSP) to negatively charged phospholipid liposomes

Magnus Nord, Jan-Åke Gustafsson, Johan Lund*

Department of Medical Nutrition, Karolinska Institute, Stockholm, Sweden

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Abstract To investigate interactions between the polychlorinated biphenyl-binding protein uteroglobin and phospholipids, we used a liposome-pelletting assay. PCB-BP/uteroglobin bound to liposomes made from negatively charged phospholipids (PtdSer and PtdIns) in the presence of 5 mM calcium. No binding to liposomes made from phospholipids without net charge (PtdChol and PtdEtn) was observed, nor could we detect binding in the absence of calcium or when magnesium was substituted for calcium. This suggests that PCB-BP/uteroglobin can bind to phospholipids in vivo and may have a role in the phospholipid homeostasis of the airway and/or secretory pathway of the Clara cell.

Key words: Uteroglobin; Polychlorinated biphenyl; Carrier protein; Calcium-binding protein; Calcium; Phospholipid

1. Introduction

Rabbit uteroglobin [1] and the homologous rat and human proteins are low molecular mass homodimeric proteins that bind metabolites of polychlorinated biphenyls (PCBs) with high affinity [2–4]. Thus, they are also known as PCB-binding proteins (PCB-BPs). Moreover, commonly used names are Clara cell secretory protein (CCSP) and Clara cell 10-kDa protein (CC10) since they are secreted in large amounts from the nonciliated Clara cell in the distal airway epithelium [5–8]. PCB-BP/uteroglobin is a major constituent of the lining fluid in the distal conducting airways [9]. In uterus, high level expression is seen in the endometrial cells during the luteal phase of the human menstrual cycle [10]. Furthermore, PCB-BP/uteroglobin is expressed during early pregnancy in rabbit [1]. The protein has also been identified in male reproductive organs in rabbit and human [11,12].

The structure of PCB-BP/uteroglobin has been extensively studied and three-dimensional structures based on X-ray diffraction data exist for the rabbit, rat and human protein [13–15]. Although several hypotheses exist regarding the physiological role of PCB-BP/uteroglobin (recently reviewed by Miele et al., 1994 [16]), a clear in vivo role has yet to be determined. In addition to binding metabolites of polychlorinated biphenyls [2–4], it also binds progesterone, although with lower affinity [17–19]. Furthermore, we have recently shown that human PCB-BP/uteroglobin binds calcium [20]. The putative calcium-binding site of PCB-BP/uteroglobin shows similarities to the calcium-binding site of secretory phospholipase A_2s ($sPLA_2$s; Barnes et al., manuscript in prep.) and is, thus, also similar to calcium-binding sites in proteins from the annexin family [21]. In $sPLA_2$s, calcium binding is necessary for enzyme activity and in annexins it mediates calcium-dependent phospholipid interactions (for recent reviews, see, e.g. Dennis et al., 1994 [22], and Raynal et al., 1994 [23]). Interestingly, phospholipids were recently found inside the hydrophobic cavity of PCB-BP/uteroglobin isolated from human sources [15]. In light of these findings, we wanted to investigate whether human PCB-BP/uteroglobin can interact with phospholipid bilayers. To this end, we used a liposome-pelleting assay previously used to characterize annexin–phospholipid interactions.

2. Materials and methods

2.1. Chemicals

All chemicals were purchased from Merck (Germany) unless otherwise stated.

2.2. Plasmids and bacterial expression

Expression of recombinant human PCB-BP/uteroglobin was carried out as previously described [20]. Briefly, the coding sequence for human PCB-BP/uteroglobin (kindly provided by Dr. G. Suske, Institut für Molekularbiologie und Tumorforschung, Marburg, Germany) was subcloned in an expression plasmid containing both lac and T7 promoters. Included was also a 3′-extension encoding four histidines. Recombinant protein was expressed in the *E. coli* strain BL21(DE3) and the His^4-tagged protein was purified via chromatography on a Ni^{2+}-Sepharose (Pharmacia Biotech, Sweden) column followed by FPLC. Trace metal ions were removed by treatment with 0.5 g chelex (BioRad, CA) per 100 mg protein. The His^4 tag did not change the phospholipid-binding characteristics as judged by comparison to non-tagged recombinant human PCB-BP/uteroglobin (data not shown).

2.3. Liposome preparation and pelletting assay

Liposomes were prepared according to Jost et al. (1994) [24]: phospholipids (purchased in chloroform or chloroform : methanol solution from Sigma, MO) were dried down under N_2 and resuspended in 40 μM EGTA. After sonication, liposomes were pelleted at 200,000 × *g* for 20 min, resuspended in 40 μM EGTA at a concentration of 7.5 mg/ml and stored under N_2. The pelleting assay, modified from Jost et al. (1994) [24], was carried out as follows: 50 mg protein was preincubated in 90 μl of 50 mM imidazole-HCl (pH 7.5), 150 mM NaCl and either 5 mM $CaCl_2$, 5 mM $MgCl_2$ or 10 mM EDTA for 20 min at 20°C with shaking. Liposomes were added to a final concentration of 3 mg/ml and incubated with shaking for an additional 20 min. Liposomes and bound protein were then pelleted at 200,000 × *g* for 20 min at room temperature. Bound protein was subsequently eluted from the pellet with a buffer containing imidazole and NaCl as above and 100 mM EDTA (20 min with shaking at 20°C). Remaining liposomes were

*Corresponding author. *Present address:* Department of Medical Nutrition, F 60 NOVUM, Huddinge University Hospital, S-141 86 Huddinge, Sweden. Fax: (46) (8) 711 66 59.
E-mail: johan.lund@mednut.ki.se

Abbreviations: BAL, bronchoalveolar lavage; BL, bronchial lavage; CC10, Clara cell 10-kDa protein; CCSP, Clara cell secretory protein; FPLC, fast protein liquid chromatography; M_r, approximate molecular weight; PAGE, polyacrylamide gel electrophoresis; PCB, polychlorinated biphenyl; PCB-BP, polychlorinated biphenyl-binding protein; PtdChol, phosphatidyl choline; PtdEtn, phosphatidyl ethanolamine; PtdIns, phosphatidyl inositol; PtdSer, phosphatidyl serine; $sPLA_2$, secretory phospholipase A_2.

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pelletted as before and the supernatants were loaded onto SDS-PAGE gels (without reducing agent). The gels were analysed through Coomassie brilliant blue G250 staining (Serva, Germany) [25].

2.4. Phospholipid-binding assay

Phosphatidyl inositol liposomes with tritiated phosphatidyl inositol (Amersham Life Sciences, UK) included to a specific activity of 2.5 Ci/mol were prepared as described in section 2.3. Recombinant protein was then incubated with increasing concentrations of phopspholipid in 50 mM imidazole-HCl (pH 7.5), 150 mM NaCl, 5 mM $CaCl_2$, 1 mM DTT and 1 mg/ml ovalbumin (Sigma). To correct for unspecific binding, incubations without recombinant protein were carried out in parallel. After overnight incubation at 37°C with shaking, samples were transferred to ice. EDTA was added to a concentration of 10 mM to brake liposome–protein interactions and liposomes were pelletted at 200,000 × *g* at 4°C. Radioactivity in the supernatant was then determined by scintillation counting. Non-pellettable, i.e. non-liposomal, PtdIns then represents total protein-bound phospholipid as well as free phospholipids in solution. Thus, differences in radioactivity between incubations with and without recombinant human PCB-BP/uteroglobin will represent specific binding of PtdIns to the protein. PtdIns concentration in the supernatant was plotted as a function of PtdIns concentration in the incubation before pelletting and linear regression analysis was used to fit straight lines for incubations with and without recombinant PCB-BP/uteroglobin. Other incubation temperatures tested (4, 20 and 30°C) did not affect the result (data not shown). Finally, incubations (without ovalbumin added) were analysed by 5–20% (w/v) linear sucrose gradients as described previously [18]. Standard proteins (aldolase, ovalbumin and cytochrome c; all from Sigma) as well as recombinant PCB-BP/uteroglobin were run in parallel to determine the expected position of radiolabelled PtdIns bound to PCB-BP/uteroglobin.

3. Results

3.1. Binding of PCB-BP/uteroglobin to phospholipid liposomes

To test whether human PCB-BP/uteroglobin can bind to phospholipid liposomes, we incubated recombinant human PCB-BP/uteroglobin with liposomes prepared from either phosphatidyl choline (PtdChol), phosphatidyl serine (PtdSer), phosphatidyl ethanolamine (PtdEtn) or phosphatidyl inositol (PtdIns). Subsequently, liposomes were pelletted and bound protein was eluted and analysed by SDS-PAGE and Coomassie blue staining. The incubations were done in the presence of either 5 mM Ca^{2+} or 10 mM EDTA. As seen in Fig. 1, PCB-BP/uteroglobin binds to negatively charged phospholipid liposomes (PtdSer and PtdIns) but not to phospholipids without net charge (PtdChol and PtdEtn). The binding is calcium-dependent and very little binding occurs in the presence of EDTA (Fig. 1). Calcium-dependent binding of PCB-BP/uteroglobin to liposomes made from a mixture of PtdChol, PtdEtn, PtdIns and lyso-PtdChol (4:3:2:1) was also observed (data not shown). No binding of PCB-BP to liposomes was detected when only μM concentrations of calcium were included (data not shown).

We next wanted to test the cation dependency of liposome binding. Therefore, PCB-BP/uteroglobin was incubated with phosphatidyl serine liposomes in either 5 mM Ca^{2+}, 5 mM Mg^{2+} or 10 mM EDTA and analysed as before. Magnesium was used since, with the exception of calcium, it is the only divalent metal ion present in mM concentration in vivo. PCB-BP/uteroglobin did not bind to phosphatidyl serine liposomes in the presence of magnesium ions (Fig. 2, lane 3). As before, PCB-BP/uteroglobin bound to phosphatidyl serine liposomes in the presence of calcium but not in the presence of EDTA (Fig. 2, lanes 4 and 2).

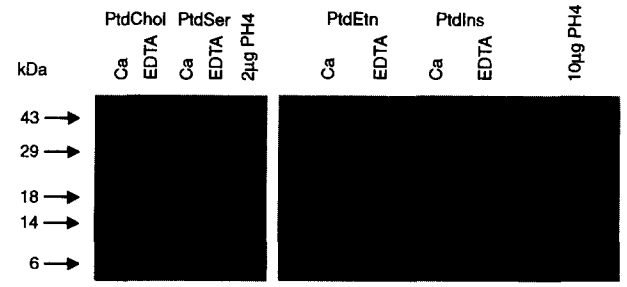

Fig. 1. Calcium-dependent binding of PCB-BP/uteroglobin to negatively charged phospholipid liposomes but not to phospholipids without net charge. 2 and 10 μg recombinant human PCB-BP were included on the gels as positive controls (PH4). Positions of molecular weight markers indicated at left.

3.2. Binding of phosphatidyl inositol to PCB-BP/uteroglobin

The observed association of human PCB-BP/uteroglobin to phosphatidyl inositol liposomes prompted us to investigate if this might be related to the binding of single PtdIns molecules inside human PCB-BP/uteroglobin [15]. One possibility might be that the physical interaction of the protein with the liposome would allow it to extract phospholipids into its ligand-binding cavity. We, therefore, designed experiments where we incubated recombinant human PCB-BP/uteroglobin with liposomes made from radiolabelled PtdIns under conditions where PCB-BP/uteroglobin binds to PtdIns liposomes. After pelletting, the liposomes in the presence of EDTA, which disrupts PCB-BP/uteroglobins interaction with the liposome, radioactivity in the supernatant will represent total protein-bound PtdIns as well as free non-liposomal PtdIns. Comparisons between incubations with and without recombinant human PCB-BP/uteroglobin then provide an estimate of PCB-BP/uteroglobin-specific binding. As shown in Fig. 3A, no additional retention of PtdIns in the supernatant can be seen in the presence of 10 μM recombinant human PCB-BP/uteroglobin (compare filled and empty circles), thus, suggesting that binding of PtdIns to PCB-BP/uteroglobin does not take place under these conditions. To verify this, the supernatant indicated in Fig. 3A was subjected to a linear sucrose gradient. This revealed no increased radioactivity in the fractions where PCB-BP/uteroglobin is expected to accumulate (Fig. 3B), further indicating that no binding of phosphatidyl inositol to human PCB-BP/uteroglobin occurs under the conditions applied here.

4. Discussion

In the present study, we show that human PCB-BP/uteroglobin can bind to anionic phospholipid liposomes in a calcium-dependent manner. The presence of negatively charged phospholipids in the liposomes is required. The binding is seen only with calcium concentrations in the mM range and magnesium cannot substitute for calcium.

The putative calcium-binding site of PCB-BP/uteroglobin shows similarities to calcium-binding sites of the phospholipid interacting secretory PLA_2s (Barnes et al., manuscript in prep.) and annexins [21]. Annexins bind to negatively charged phospholipids in the presence of μM calcium [23]. In contrast, PCB-BP/uteroglobin needs calcium concentrations in the mM range. Calcium concentrations in the lining fluid of conducting airways has been reported to be around 4 mM in humans [26].

Thus, PCB-BP/uteroglobin interaction with anionic phospholipids may very well be possible in the airway lining fluid of the small airways. Studies of the subcellular localization of PCB-BP/uteroglobin in Clara cells show the protein to be present preferentially in endoplasmic reticulum and in secretory granules [6]. Calcium concentrations within the endoplasmic reticulum are considered to be approximately 2 mM, whereas secretory granules often contain higher concentrations (typically 20–40 mM; see Meldolesi et al., 1990 [27], and references therein). Thus, the potential for phospholipid interactions exists in these organelles as well. Moreover, PCB-BP/uteroglobin has recently been shown to bind to microsomes and plasmatic membranes. A different mechanism seems involved in this case, as PCB-BP/uteroglobin was associated with binding component(s) with an apparent M_r of 90,000, suggesting the presence of a membrane-associated uteroglobin-binding protein [28]. Nevertheless, it is noteworthy that these experiments were carried out in buffers containing 5 mM calcium.

Negatively charged phospholipids (PtdSer and PtdIns) seem to be more abundant in the lining fluid of the conducting airways than in the lining fluid of the alveoli, as judged from analysis of phospholipids in human bronchial lavage (BL) and bronchoalveolar lavage (BAL). PtdSer and PtdIns comprise approximately 25% of total phospholipids in BL and only 5% of total phospholipids in BAL [29]. Thus, PCB-BP/uteroglobin binding to phospholipids appears more likely in the conducting airways than in the alveolar lining, i.e. the pulmonary surfactant, where phosphatidyl choline predominates [30]. This is in agreement with data from our laboratory showing that PCB-BP/uteroglobin cannot be detected in surfactant preparations of rat lung (Nord et al., unpubl. data).

One described property of PCB-BP/uteroglobin is its anti-inflammatory effects. These have primarily been correlated to PCB-BP/uteroglobin's ability to inhibit secretory PLA_2 in vitro [reviewed in 16]. Since PLA_2s are considered to act optimally when the phospholipid substrate is part of a lipid–water interface, as for instance a membrane [22], the binding of PCB-BP/uteroglobin to phospholipid bilayers might allow it to interact with PLA_2s at their site of action. Besides direct PCB-BP/uteroglobin-PLA_2 interactions, the binding of PCB-BP/uteroglobin to phospholipids and/or the binding of phospholipids inside the protein [15] offers a potential mechanism for PLA_2 inhibition by substrate blockage/sequestring. However, in studies on the inhibition of secretory PLA_2s by PCB-BP/uteroglobin from our group and others [20 and references therein], this does not seem to be the case, as increasing the amount of phospholipid substrate does not overcome the inhibition. Moreover, our study also shows that the in vitro inhibition is a consequence of the calcium-binding activity of PCB-BP/uteroglobin [20]. The protein sequesters calcium, an essential cofactor for $sPLA_2$s and no inhibition occurs in the presence of mM amounts of calcium. Still the potential in vivo colocalization of PCB-BP/uteroglobin with PLA_2s at phospholipid membranes might allow local effects on calcium concentrations. In conclusion, the anti-inflammatory/PLA_2 inhibitory properties of PCB-BP/uteroglobin are complex and not completely understood. Investigating the structure–function relationships of PCB-BP/uteroglobin, especially with regard to its calcium- and phospholipid-binding properties, seems especially important to further our understanding in these matters.

Recently, phosphatidyl inositol and phosphatidyl choline was found inside the hydrophobic cavity of PCB-BP/uter-

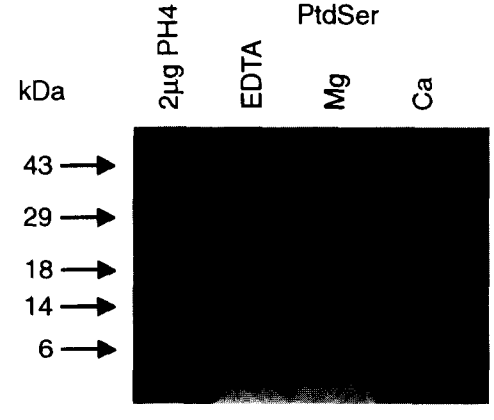

Fig. 2. Binding of PCB-BP/uteroglobin to negatively charged phospholipid liposomes is dependent on calcium but not magnesium. 2 µg recombinant human PCB-BP was included on the gel as a positive control (PH4). Positions of molecular weight markers indicated at left.

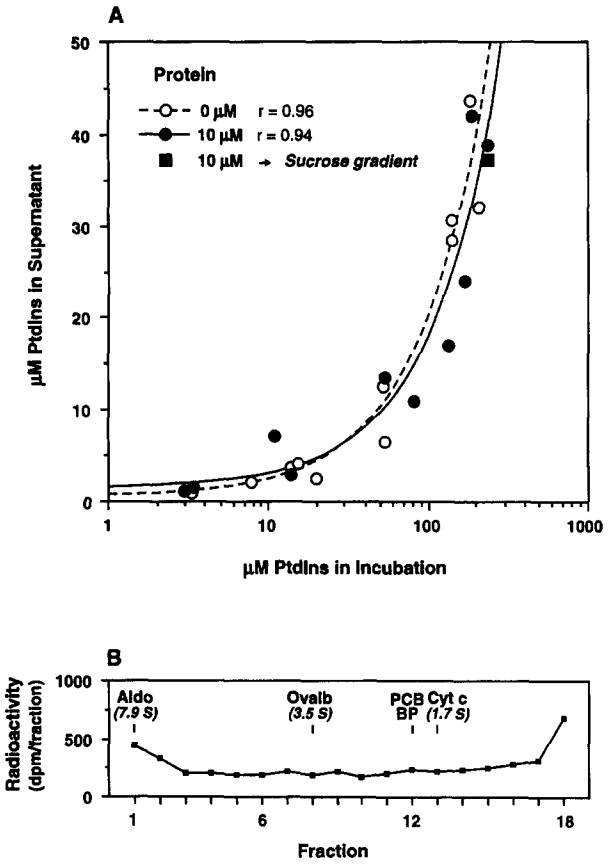

Fig. 3. PCB-BP/uteroglobin does not bind phosphatidyl inositol. (A) Non-liposome-associated PtdIns as a function of PtdIns added to the incubation (presented as a lin-log graph for a clearer view of low PtdIns concentrations). Straight lines were fitted by linear regression to points representing experiments with (filled circles) and without (empty circles) 10 µM recombinant human PCB-BP/uteroglobin. *R* values are indicated for each fit. Also indicated is the point analysed by sucrose gradients in B. (B) Quantification of radioactive PtdIns in 5–20% linear sucrose gradients. Indicated are the positions of standard proteins and their respective *S* values, as well as the position of recombinant PCB- BP/uteroglobin.

oglobin isolated from human sources [15]. Access to this hydrophobic cavity probably requires reduction of the disulfide bridges in the protein. Reduction has been suggested to allow conformational changes that might mediate interactions with lipid membranes [15], providing a mechanism for conformationally regulated uptake of phospolipids. The calcium-dependent binding of PCB-BP/uteroglobin to phospholipid bilayers might then provide further mechanisms for the protein to get in close proximity and interact with its potential ligand. Nevertheless, we could not uncover any binding of PtdIns to human PCB-BP/uteroglobin under the conditions allowing PCB-BP/uteroglobin to bind to liposomes that we describe here. Since the PtdIns concentration in epithelial lining fluid of human airways has been reported to be around 60 μM [31] and most of epithelial lining fluid phospholipids is in the form of surfactant, the conditions tested here (PtdIns presented in the form of liposomes in concentrations ranging from 5 to 300 μM) appear physiologically relevant. Other attempts by our laboratory to study the binding of PtdIns to human PCB-BP/uteroglobin has included direct binding assays using other vehicles than liposomes to deliver the phospholipid, as well as competition assays using PtdIns in liposomes to compete with binding of radiolabelled progesterone. However, none of these assays have been successful in showing binding of PtdIns to PCB-BP/uteroglobin (data not shown). One possibility is that the binding of phosphatidyl inositol in the ligand-binding cavity may require unknown additional factor(s) which are normally present in vivo.

In conclusion, our study shows that PCB-BP/uteroglobin can bind to phospholipid liposomes under conditions present in the conducting airways and intracellularly in endoplasmic reticulum and secretory granules. Thus, it provides a mechanism whereby PCB-BP/uteroglobin can get in close proximity to phospholipid bilayers in vivo. Even though we have been unable to demonstrate binding of phosphatidyl inositol to PCB-BP/uteroglobin in vitro, these data together with the finding of phospholipids inside the hydrophobic cavity of PCB-BP/uteroglobin [15] suggest a role for PCB-BP/uteroglobin in the phospholipid homeostasis of the airways and/or the secretory pathway of the Clara cell.

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