New lipid interaction partners stimulate the inhibition of activated protein C by cell-penetrating protein C inhibitor

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Summary
Protein C inhibitor (PCI, SerpinA5) is a heparin-binding serpin which can penetrate through cellular membranes. Selected negatively charged phospholipids like unsaturated phosphatidylserine and oxidised phosphatidylethanolamine bind to PCI and stimulate its inhibitory activity towards different proteases. The interaction of phospholipids with PCI might also alter the lipid distribution pattern of blood cells and influence the remodelling of cellular membranes. Here we showed that PCI is an additional binding partner of phosphatidic acid (PA), cardiolipin (CL), and phosphoinositides (PIPs). Protein lipid overlay assays exhibited a unique binding pattern of PCI towards different lipid species. In addition PA, CL, and unsaturated, monophosphorylated PIPs stimulated the inhibitory property of PCI towards activated protein C in a heparin like manner. As shown for kallistatin (SerpinA4) and vaspin (SerpinA12), the incubation of cells with PCI led to the activation of protein kinase B (AKT), which could be achieved through direct interaction of PCI with PIPs. This model is supported by the fact that PCI stimulated the PIP-dependent 5-phosphatase SHIP2 in vitro, which would result in AKT activation. Hence the interaction of PCI with different lipids might not only stimulate the inhibition of potential target protease by PCI, but could also alter intracellular lipid signalling.

Keywords
Protease inhibitors, phospholipids, coagulation factors, signal transduction, SERPINs

Introduction
Protein C inhibitor (PCI, SerpinA5) is a serpin that exhibits a localisation duality. In the vascular system PCI is mainly produced and secreted by the liver (1). In addition to its presence in the vasculature, PCI can be detected in many other biological fluids and influences fertility and cancer development (2-5). Like many other serpins, PCI possesses the ability to bind to non-protease ligands like glycosaminoglycans, DNA (6), retinoic acid (7), or phospholipids (8, 9) which might either stimulate its activity (10) or contribute to other yet unknown biological effects.

In addition to the stimulatory effect of negatively charged phospholipids on protease inhibition by PCI, we demonstrated previously (11) that the interaction of PCI with phosphatidylethanolamine (PE) leads to the uptake of the serpin into live cells. It was shown that biotin-labelled PCI is efficiently taken up by human leukocytic HL-60 cells both at 37°C and at 6°C within 10 minutes (min) of incubation (11). Interestingly the translocation of PCI across cellular membranes leads to accumulation of the serpin in the peri- and intranuclear compartments. The observed nucleocytoplasmatic localisation of PCI might suggest the presence of a cryptic nuclear localisation signal (NLS), the interaction with a NLS containing binding partner or another mechanism leading to nuclear translocation of PCI. In vivo experiments show similar results, as injection of biotin-labelled PCI into the circulation of mice, leads to accumulation of the serpin in leukocytes. Further analysis of the leukocytic pool revealed that PCI integrates in the nuclei of cells from the granulocyte fraction (11).

As mentioned above, phospholipids like PE mediate the uptake of PCI into blood cells. Up to now, neither the exact mechanism and prerequisites of internalisation, nor the functional role of internalised PCI are known. To clarify that situation, our group is working on the identification of novel intracellular interaction partners of cytosolic and nuclear PCI. Other phylogenetically related serpins from the A-clade family like kallistatin (SerpinA4) or vaspin (SerpinA12) have been shown to activate the phospholipid-binding protein kinase B (AKT) in a phosphoinositide 3-kinase (PI3K) dependent manner and to protect endothelial cells from apoptosis (12, 13). The exact mechanism how this is achieved has still to be clarified. Since PCI interacts with negatively charged ligands and can be taken up by cells in a phospholipid dependent manner, we ask the question whether internalised PCI affects lipid signalling by interacting directly with phospholipids. Here we analysed the interaction of PCI with different lipid species like fatty...
acids, diacylglycerol (DAG), phosphatidic acid (PA), cardiolipin (CL) or phosphoinositides (PIPs). PIPs affinity for phospholipids might alter the cellular lipid distribution pattern and influence the remodelling of cellular membranes (14). Here the direct interaction of PCI with intracellular phospholipids and lipid second messengers is put forward as new mechanism for the action of internalised PCI.

PIPs are a group of negatively charged phospholipids comprising only several percent of intracellular lipids. They are essential components of eukaryotic membranes and regulate a repertoire of fundamental biological processes (15). PIPs consist of a membrane anchored DAG backbone linked to a myo-inositol head group via a phosphodiester bond. The headgroup is easily accessible for cytosolic proteins and may be phosphorylated at position 3, 4 or 5 giving rise to seven different PIPs. The rapid interconversion of PIPs is regulated by several PIP-specific kinases and phosphatases catalysing more than 18 different reactions (16).

Intracellular levels of phosphatidylinositol 3,4,5-trisphosphate (PtdIns(3,4,5)P$_3$) act as important second messenger, recruiting signalling molecules like AKT to the cell membrane (17). Dephosphorylation of PtdIns(3,4,5)P$_3$ is needed to terminate AKT signalling. This is catalysed in vivo by 3-kinases like PTK (phosphatase and tensin homologue deleted on chromosome 10) forming phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P$_2$). The dephosphorylation at position 5 on the other hand leads to the generation of phosphatidylinositol 3,4-bisphosphate (PtdIns(3,4)P$_2$) (18), which might also contribute to the activation of AKT (19). Among the 10 mammalian inositol 5-phosphatases (20), SH2 (Src homology 2) domain containing inositol phosphatase 2 (SHIP2) is known as a negative regulator of insulin signalling (21). In contrast to SHIP1 which is mainly present in haematopoietic cells, SHIP2 is expressed in many human tissues and different cell types (22). As expected, insulin-dependent activation of AKT is enhanced in SHIP2-deficient mice, but surprisingly these mice are resistant to dietary weight gain (23).

Here, we determined the fate of AKT following overexpression of non-secreted PCI in HEK293-cells. Furthermore, the capacity of PIPs to bind directly to PCI and to modulate its inhibitory property towards APC in a heparin-like manner was investigated. In addition, in vitro experiments were performed to determine whether PCI might influence the phosphatase activity of the PIP-dependent 5-phosphatase SHIP2.

Materials and methods

Materials

Expression and purification of recombinant human PCI (rhPCI) using a bacterial expression system was performed as described previously (24). Purification of PCI from human plasma (pPCI) was done as described (8). Fully glycosylated recombinant human PCI (gPCI) was obtained from Novoprotein (Shanghai, China). Monoclonal mouse anti-human SerpinA5 IgG, and monoclonal mouse anti-human SerpinA1 IgG were obtained from R&D Systems (Minneapolis, MN, USA). Monoclonal mouse anti-AT3 IgG was from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Mouse anti-Penta-His IgG was from Qiagen (Hilden, Germany). Monoclonal rabbit anti-AKT IgG, and monoclonal rabbit anti-pAKT(T308) IgG were from Cell Signaling Technology (Danvers, MA, USA). Polyclonal HRP-conjugated sheep anti-mouse IgG, polyclonal HRP-conjugated donkey anti-rabbit IgG, and Tween-20 were from GE Healthcare (Vienna, Austria). Rabbit anti-actin IgG, purified human plasma antithrombin (AT3), purified human plasma a1-antitrypsin (A1AT), skim milk powder, NaCl, CaCl$_2$, KCl, HEPES, Tris, bromophenol blue, and glycerol were from Sigma-Aldrich (St. Louis, MO, USA). APC and heparin were gifts from Baxter (Vienna, Austria). BSA was from Bioool (Hamburg, Germany). Chromogenic substrate S-2366 was from Chromogenix (Milano, Italy). SuperSignal West Femto chemiluminescent substrate was from Thermo Fisher Scientific (Vienna, Austria). Stearic acid, arachidonic acid, 1-palmitoyl-rac-glycerol, dipalmitoylglycerol and 1-stearoyl-2-arachidonoyl-glycerol were from Sigma-Aldrich. Dipalmitoyl-PtdIns3P, dipalmitoyl-PtdIns4P, dipalmitoyl-PtdIns5P, and dioleoyl-PtdIns(3,4,5)P$_3$ were from Cayman Europe (Tallinn, Estonia). 1-palmitoyl-2-arachidonoyl-PS, dipalmitoyl-PA, 1-palmitoyl-2-arachidonoyl-PA, 1-stearoyl-2-arachidonoyl-PtdIns(3,4)P$_2$, 1-stearoyl-2-arachidonoyl-PtdIns(4,5)P$_2$, tetraoleoyl-CL, coenzyme A (CoA), palmitoyl-CoA, stearoyl-CoA and arachidonoyl-CoA were from Avanti polar lipids (Alabaster, AL, USA).

Cell lines and transfection

The human prostate carcinoma cell line LNCaP-FGC (ATCC), which is referred to in this paper as LNCaP, was cultured in RPMI-1640 supplemented with 10% fetal bovine serum (FBS, Sigma-Aldrich), 50 units/ml penicillin and 50 µg/ml streptomycin. RWPE-1 cell line (ATCC) was cultured in Keratinocyte Serum Free Medium (K-SFM, Life Technologies - Gibco, Vienna, Austria) supplemented with bovine pituitary extract (BPE) and human recombinant epidermal growth factor (EGF). Human embryonic kidney cells (HEK293) were obtained from the collection of the Department of Vascular Biology and Thrombosis Research, and were cultured in DMEM (Sigma-Aldrich) supplemented with 10% fetal bovine serum (FBS) in a humidified incubator with 5% CO$_2$ at 37°C. Primary human umbilical vein endothelial cells (HUVECs) were a kind gift from Dr. Johannes Schmid (Department of Vascular Biology and Thrombosis Research, Vienna, Austria). HUVECs were cultured in M199 (Sigma-Aldrich) supplemented with 10% FBS, 30 µg/ml endothelial cell growth supplement, 90 µg/ml heparin, 50 units/ml penicillin and 50 µg/ml streptomycin in a humidified incubator with 5% CO$_2$ at 37°C and used between passage 3 and 5.

LNCaP and RWPE-1 cells were transiently transfected by Lipofectamine LTX and Plus reagent (Life Technologies - Invitrogen, Vienna, Austria) according to instructions of the manufacturer. In brief, cells were grown on Permanox slides (Nunc, Roskilde, Denmark) and transfected with appropriate vectors. At 48 hours (h) after the transfection, cells were fixed with 3% paraformaldehyde in phosphate-buffered saline (PBS) for 20 min at room tempera-
tured followed by washing with PBS. Confocal images were taken by Zeiss LSM-Meta microscope system with a plan apochromat 63x/1.4 DIC oil immersion objective.

PCI-mRNA lacking the sequence corresponding to the signal peptide was cloned into pCMV-Myc vector (Clontech, Mountain View, CA, USA) between EcoRI and SalI restriction sites. Transfection of cells with this vector leads to the expression of non-secreted PCI carrying a Myc-tag at the N-terminus. The construct was taken from the plasmid collection of the Department of Vascular Biology and Thrombosis Research (Vienna, Austria). HEK293 cells were transfected using a calcium-phosphate method. As control, cells were also transfected with empty pEGFP-N1 (Clontech) leading to the expression of EGFP (enhanced green fluorescent protein) under CMV-promotor.

HUVECs were seeded into 12-well plates (Nunc, Roskilde, Denmark) coated with 1% gelatin (Sigma-Aldrich). Confluent wells were washed with PBS and then starved for 2 h in M199 without any supplements followed by 15 min incubation with 100 nM human insulin (Sigma-Aldrich) or 200 nM fully glycosylated recombinant human PCI (gPCI, Novoprotein) in M199. Thereafter cells were lysed in sample buffer containing 10% β-ME and analysed by Western blotting.

**Protein lipid overlay assays**

For quantitative dot blots, 0.5 nmol of the indicated lipid species dissolved in mixtures of different organic solvents according to manufacturer's instructions were spotted onto PVDF membranes (Carl Roth, Karlsruhe, Germany). Thereafter membranes were treated as described below for PIP-Strips and PIP-Arrays. PIP-Strips and PIP-Arrays (Echelon Biosciences Incorporated, Salt Lake City, UT, USA) were used according to the manufactures instructions. In brief, blocking of the membrane was performed using 3% BSA in PBS at room temperature (RT) for 1 h. The blocking solution was discarded and the membrane was incubated with 1 µg/ml PCI, A1AT or AT3, respectively, for 1 h at RT in PBS containing 3% BSA. The membrane was washed three times with PBS containing 0.1% Tween-20 (PBST) for 10 min each. Thereafter the membrane was incubated with monoclonal mouse anti-human SerpinA5 IgG (0.5 µg/ml), monoclonal mouse anti-human SerpinA1 IgG (1 µg/ml) or monoclonal mouse anti-AT3 IgG (0.2 µg/ml), respectively, for 1 h at RT in PBS with 3% BSA. After washing the dot-blots three times for 10 min with PBST, secondary polyclonal HRP-conjugated sheep anti-mouse IgG (1:5,000) was applied followed by three washing steps (10 min each) with PBST. The signal was detected with SuperSignal West Femto chemiluminescent substrate using a FluorChem HD2 imaging system (Alpha Innotech, Cell Biosciences, Santa Clara, CA, USA).

**Preparation of phospholipid vesicles**

Phosphoinositides and other phospholipids were dissolved in mixtures of different organic solvents according to manufacturer's instructions and stored at -70°C. For each experiment different amounts of phospholipids were put into 1.5 ml Eppendorf tubes. The organic solvent was evaporated under shaking using a constant argon stream to produce a thin phospholipid layer on the wall of the tube. Dried phospholipids were resuspended in pre-warmed buffer (37°C) by vortexing at maximal speed for 60 seconds followed by shaking on a thermomixer for 5 min at 37°C and 1,100 rpm.

**Native PAGE, SDS-PAGE and Western Blotting**

The interaction of recombinant PCI with unsaturated PtdIns(3,5)P2 and PtdIns(4,5)P2 was studied by native polyacrylamide gel electrophoresis (PAGE) followed by Western blotting as described previously with minor modifications (8). PCI (400 ng) was incubated in a volume of 16 µl without or with PtdIns(3,5)P2 or PtdIns(4,5)P2 (50 µM each) in PBS for 30 min at 37°C. Thereafter, an equal volume of sample buffer (23% glycerol, 0.01% bromphenol blue in 125 mM Tris-HCl, pH 6.8) was added and the samples were analysed on a 10% native acrylamide gel followed by Western blotting. Proteins were transferred onto PVDF membranes (Carl Roth). The membrane was blocked with 5% BSA in PBST for 1 h at RT. The membrane was incubated with mouse anti-Penta-His IgG (0.1 µg/ml) for 1 h at RT, and washed five times (5 min each) with PBST prior to the addition of HRP-conjugated sheep anti-mouse IgG (1:1,000). The signal was detected with SuperSignal West Femto chemiluminescent substrate using a FluorChem HD2 imaging system.

SDS-PAGE followed by Western blotting of HEK293 cell lysates and HUVEC lysates was performed according to the method of Laemmli (25) using 10% acrylamide gels. PCI was detected using monoclonal mouse anti-SerpinA5 IgG (0.5 µg/ml). AKT and pAKT(T308) levels were detected using monoclonal rabbit anti-AKT IgG (1:1,000) and monoclonal rabbit anti-pAKT(T308) IgG (1:1,000), respectively. Rabbit anti-actin IgG (1:1,000) was used to detect actin. The respective secondary antibodies were HRP-conjugated sheep anti-rabbit IgG (1:5,000), and HRP-conjugated sheep anti-mouse IgG (1:5,000). The signal was detected using SuperSignal West Femto chemiluminescent substrate with a FluorChem HD2 imaging system.

**Inhibition of APC by PCI**

Inhibition of APC by PCI was tested on 96-well microtitre plates (Greiner Bio-One, Kremsmünster, Austria). APC (0.1 nM f.c.) was incubated with PCI (10 - 40 nM f.c.) in the absence or presence of heparin (5 U/ml f.c.) phospholipids, activated fatty acids or CoA in 100 µl of APC-buffer (25 mM HEPES, 137 mM NaCl, 3.5 mM KCl, 3 mM CaCl2, 1% BSA, pH 7.4). After 60 min of incubation at 37°C, 100 µl of the chromogenic substrate S-2366 (0.4 mM) dissolved in APC-buffer, was added to each well. The colour development was monitored with a Synergy H4 microplate reader (BioTek, Seattle, WA, USA) at 405 nm. The 30 min timepoint was used to calculate the relative activity. The amidolytic activity of APC in the absence of PCI was assigned to 1, and for each PCI concentration in combination with or without heparin and phospholipids, the remaining APC activity was calculated. In the ab-
sence of PCI, the activity of APC was not affected by phospholipids or heparin.

**Activity assay for SHIP2**

SHIP2 phosphatase activity in the presence or absence of PCI was determined on 96-well plates by measuring phosphate release from PtdIns(3,4,5)P$_3$ using an acidic malachite green phosphate detection kit (BioAssay Systems, Hayward, CA, USA) as described previously (26). Soluble dioleoyl-PtdIns(3,4,5)P$_3$ was dissolved in water at 1 g/l and stored in aliquots at -70°C until further usage. Seventy-five µM PtdIns(3,4,5)P$_3$ was preincubated with 37.5, 75, 150 or 300 nM PCI purified from human plasma in 40 µl assay buffer for 10 min. The reaction was initiated by adding 20 µl of 15 nM recombinant human SHIP2 (Echelon Biosciences, Salt Lake City, UT, USA) diluted in assay buffer. Samples were incubated for 1 h at 37°C, and the reactions were stopped by the addition of 15 µl malachite green reagent. The samples were left for 10 min at RT for colour development before measuring the absorbance at 620 nm with a Synergy H4 microplate reader. Phosphate release was quantified using a standard curve of K$_2$HPO$_4$ in assay buffer.

**Results**

We analysed the interaction of the inhibitory serpin PCI with different lipid species. ►Figure 1 and ►Figure 2 provide the reader with dot blot analysis illustrating the specific binding pattern of PCI towards various phospholipids and other potential lipid ligands. In addition the modulatory potential of lipid species that exhibited positive signals in dot blot analysis was determined using the inhibition of APC by PCI as model system (see ►Figures 3-5). ►Figure 6 and ►Figure 7 deal with the influence of PCI on lipid signalling and provide data on the stimulatory effect of PCI on protein kinase B (AKT) activation and on the activity of the phosphoinositide-specific phosphatase SHIP2.

**PCI binds to diacylglycerol, phosphatidic acid and cardiolipin**

Since PCI is known to bind classical membrane phospholipids like phosphatidylserine (PS) and phosphatidylethanolamine (PE), we wanted to determine whether PCI could also bind to other phospholipids and lipid species. Recombinant human PCI was used in a qualitative dot blot assay as described to determine PCI's lipid specificity. In agreement with our previous results (8), in this experiment oxidised PA-PS (OxPA-PS) showed strong binding to PCI and was used as positive control (►Figure 1). Spots of DAG and phosphatidic acid (PA) carrying different fatty acids showed positive signals in dot blot analysis with PCI. A strong signal was also detected for the cardiolipin spots (►Figure 1). No signals could be detected with monoacylglycerol (MAG), stearic acid or arachidonic acid. CHCl$_3$ which was used as negative control also failed to show binding to PCI (►Figure 1). Hence, it would seem

<table>
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<tr>
<th>Different lipid species</th>
<th>Qualitative dot blot</th>
<th>Molecular Structure</th>
<th>Stimulation of APC inhibition</th>
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<td>CHCl$_3$</td>
<td></td>
<td>negative control</td>
<td>n.d.</td>
</tr>
<tr>
<td>OxPA-PS</td>
<td></td>
<td>positive control, see Malieier et al. 2007</td>
<td>yes</td>
</tr>
<tr>
<td>Stearic acid 18:0</td>
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<tr>
<td>Arachidonic acid 20:4</td>
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<td></td>
<td>no</td>
</tr>
<tr>
<td>16:0 MAG</td>
<td></td>
<td></td>
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<tr>
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<td></td>
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<tr>
<td>SA-DAG</td>
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<tr>
<td>16:0 PA</td>
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<tr>
<td>PA-PA</td>
<td></td>
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</tr>
<tr>
<td>OxPA-PA</td>
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<tr>
<td>Cardiolipin (tetO-CL)</td>
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**Figure 1: Specific binding pattern of PCI towards immobilised phospholipids.** 500 pmol of different lipids as indicated were spotted onto PVDF membranes and incubated with 1 µg/ml of recombinant human PCI (rhPCI) as described in Materials and methods. Thereafter the membrane was incubated with monoclonal mouse anti-human PCI IgG followed by polyclonal HRP-conjugated sheep anti-mouse IgG. Bound protein was detected with SuperSignal West Femto chemiluminescent substrate. DAG, diacylglycerol; MAG, monoacylglycerol; n.d., not determined; Ox, oxidised; PA-PA, 1-palmitoyl-2-arachidonoyl-phosphatidic acid; PA-PS, 1-palmitoyl-2-arachidonoyl-phosphatidylserine; SA-, 1-stearoyl-2-arachidonoyl; tetO-CL, tetraoleoyl-cardiolipin.
as if PCI interacted with many different lipid species, and it would be interesting to determine whether these compounds exhibit a modulatory effect on protease inhibition by PCI.

**PCI shows a specific binding pattern towards immobilised PIPs**

To test the binding potential of PCI to PIPs, a commercially available strip of nitrocellulose prespotted with the indicated phospholipids was applied, which included a blank control. The PIP strip was incubated with PCI purified from human plasma in solution. As controls, two additional heparin-binding serpins (A1AT and AT3) were also used. The signal was detected according to manufacturer’s instructions. The results revealed that PCI showed a specific binding pattern towards monophosphorylated PIPs followed by bis- and trisphosphorylated PIPs. Densitometric analysis revealed that PCI strongly bound to monophosphorylated PIPs followed by bis- and trisphosphorylated PIPs. In agreement with our previous results PCI failed to show any binding to the other phospholipids tested.

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show binding to PCI (8). Interestingly, PCI binding to PE was also not detectable in this assay, whereas the low level of binding seen with PS was determined statistically as not being significant (second last column, Figure 2B). In addition signals were absent for lyso-phosphatidylcholine (LPC). PtdIns or sphingosine-1-phosphate (S1P), and low binding was seen with lyso-phosphatic acid (LPA) which was statistically not significant as judged from densitometric analysis compared with the blank dot (Figure 2B). Contrary to our previous observations, PCI binding to PA was not detectable in this assay. The results demonstrated that both, the blank and the PC spot, did not give rise to a specific signal upon incubation with PCI, thereby verifying the experimental conditions used (Figure 2A, panel PCI). Hence, it would seem as if PCI interacted with PIPs, and it would be interesting to determine whether this could be explored using a lipid array.

**Binding of PCI with immobilised PIPs is saturable**

To examine the relative affinity of PCI to PIPs, the interaction of the serpin with PIP-Arrays, which are spotted with different concentrations of all seven PIPs as well as unphosphorylated PtdIns, was studied (Figure 2C). Densitometric analysis of the monophosphorylated PIPs revealed that the binding of PCI to PIPs was saturable (not shown). To determine the binding affinity of PCI to selected PIPs, different PIPs (50 µM) were coated on plates and incubated with different concentrations of recombinant human PCI (1.25-80 nM). Binding curves were rather flat (not shown) and half maximal binding values (K_d) for saturated PtdIns4P, PtdIns(4,5)P_2 and PtdIns(3,4,5)P_3 have been calculated to be ~0.5 µM, ~0.6 µM and ~1.6 µM, respectively.

**PCI binds to PIP vesicles in suspension**

Recombinant PCI was incubated with unsaturated SA-PtdIns(3,5)P_2 or SA-PtdIns(4,5)P_2 and thereafter analysed by native PAGE (10%) followed by Western blotting. PCI antigen showed a mobility shift, confirming binding of PCI to PtdIns(3,5)P_2 or PtdIns(4,5)P_2 in lipid suspension (Figure 2D). Interestingly no mobility shift was observed when PCI was incubated with saturated PIPs (data not shown).

**PCI’s inhibitory property is stimulated by activated fatty acids**

Free fatty acids like stearic acid or arachidonic acid that were used in this work to study PCI’s lipid specificity did neither show binding to PCI (Figure 1) nor stimulate protease inhibition. To determine the contribution of fatty acids to the stimulatory effect of phospholipids on APC-inhibition by PCI, activated fatty acids (thioesters of fatty acids with CoA) which show a high solubility

![Figure 3: Stimulatory effect of acyl-CoAs on APC inhibition by PCI.](image-url) Different acyl-CoAs at the indicated concentrations were used to stimulate the inhibition of APC (1 nM) by recombinant human PCI (rhPCI, as indicated). Heparin (5 U/ml) was used as a positive control and remaining APC activity was quantified using the chromogenic substrate S-2366 as described in Materials and methods. The activity of APC in the absence of PCI was set as 1. For each replication different preparations of recombinant human PCI were used. Data represent mean values ± SEM, n = 3. All indicated concentrations are final concentrations.
were used here. CoA alone did not exhibit any modulatory effect on APC inhibition by PCI (data not shown). Interestingly acyl-CoAs stimulated PCIs inhibitory activity towards APC in a concentration dependent manner. Among the two saturated acyl-CoAs tested, stearoyl (18:0)-CoA at 100 µM showed a comparable effect on PCI stimulation as heparin at 5 U/ml. The stimulatory effect of palmitoyl (16:0)-CoA at 100 µM was similar to the effect of 18:0-CoA at 50 µM. In addition polyunsaturated arachidonoyl-CoA did also stimulate APC inhibition by PCI (▶Figure 3).

Phosphatidic acid and cardiolipin stimulate APC inhibition by PCI

Here we tested all compounds that were used in qualitative dot blots for their ability to stimulate PCI’s inhibitory property. The inhibition of APC by PCI was used as a model system to determine the modulatory potential of each compound. Among the two saturated acyl-CoAs tested, stearoyl (18:0)-CoA at 100 µM showed a comparable effect on PCI stimulation as heparin at 5 U/ml. The stimulatory effect of palmitoyl (16:0)-CoA at 100 µM was similar to the effect of 18:0-CoA at 50 µM. In addition polyunsaturated arachidonoyl-CoA did also stimulate APC inhibition by PCI (▶Figure 3).

Phosphoinositides stimulate the inhibition of APC by PCI

As mentioned above, some negatively charged phospholipids like PA-PS, oxidised PA-PE (8) or PA-PA (▶Figure 4) stimulate the inhibition of APC and thrombin by PCI. This assay was chosen to determine whether PIPs might also have a modulatory effect on the inhibition of a potential, yet unidentified intracellular target protease by PCI. Saturated monophosphorylated PIPs slightly stimulated APC inhibition by PCI in a heparin like manner (▶Figure 5). Interestingly no effect was observed with saturated biphosphorylated PIPs or saturated PtdIns(3,4,5)P$_3$.

PCI is additionally localised in the membrane

Although PCI is considered as a secreted protein, we wanted to see whether it had additional locations in the cell. To determine PCI’s subcellular distribution, a fusion protein with EGFP that lacked the N-terminal signal peptide was overexpressed in cells, which where then studied using fluorescence microscopy. The intracellular distribution of non-secreted PCI-EGFP fusion was analysed in the denoted two prostate cell lines. In agreement with previously published data on the intracellular localisation of PCI (11), an intense signal was observed in the nucleus. Furthermore, fluorescence was additionally detected along the plasma membrane (▶Figure 6A-C). Hence, from the nuclear localisation it would appear that a cryptic nuclear localisation signal (NLS) might occur in the fusion protein or that PCI interacts with a NLS containing binding partner leading to nuclear translocation of the serpin.

Overexpression of PCI leads to the activation of AKT

As mentioned above some A-calte serpins like kallistatin or vaspin trigger protein kinase B (AKT) activation by a jet unknown mechanism. To determine whether PCI had a role in activating AKT, the serpin was overexpressed in mammalian cell lines. As a negative control, cells expressed a nonrelated EGFP. HEK293 cells were transfected with a vector expressing a non secreted version of PCI carrying a Myc-tag at the N-terminus driven by the cytomegalovirus

![Figure 4: PCI is stimulated by phosphatidic acid and cardiolipin.](https://www.thrombosis-online.com/issue/111.1/2014/figure4.png)

APC (1 nM) was incubated with different concentrations of fully glycosylated recombinant human PCI (ghPCI) or recombinant human PCI (rhPCI) purified from a bacterial expression system (as indicated) for 60 min at 37°C in the absence or presence of PA-PA or tetO-CL at the indicated concentrations. Heparin was used as a positive control at a concentration of 5 U/ml. Remaining APC activity was quantified using the chromogenic substrate S-2366 as described in Materials and methods. The activity of APC in the absence of PCI was set as 1. For each replication different preparations of human plasma PCI were used. Data represent mean values ± SEM, n = 3. All indicated concentrations are final concentrations.
promoter (CMV-Myc-iPCI), and activation levels were determined by antibodies against phosphorylated AKT. The results showed that overexpression of iPCI led to an increased signal due to the antibody detecting AKT phosphorylation, while the overexpression of EGFP under the CMV promoter in the same experiment did not cause the same effect (▶Figure 6D). To validate the observation with overexpressed PCI, AKT activation was also studied in primary HUVECs stimulated with exogenously added PCI. Cells were treated with PCI or insulin, a known activator of AKT (27), and examined for total and phosphorylated AKT levels. The results showed that the level of phosphorylated AKT in relation to total AKT was small in untreated cells (control in ▶Figure 6E-F). Stimulation with insulin led to a noticeable increase in AKT activation (centre column in ▶Figure 6E-F). Using PCI, the proportion of phosphorylated versus total AKT shifted towards activation. Densitometric analysis normalised as pAKT/tAKT revealed that addition of PCI to the medium led to an increase in AKT phosphorylation up to 1.5 fold compared to control (third column in ▶Figure 6E-F).

**PCI stimulates the activity of SHIP2 in vitro**

As described above, AKT phosphorylation was shown to be stimulated by PCI. AKT activation is not only triggered by PtdIns(3,4,5)P_3 but also by PtdIns(3,4)P_2 (19), which is mainly generated by the action of PI-dependent 5-phosphatases. Assuming that accumulation of additional active phospholipid species could occur upon stimulation of PI-dependent phosphatases, the influence of PCI on the activity of the 5-phosphatase SHIP2 was studied in the purified system. Here we used soluble PtdIns(3,4,5)P_3 as substrate for the 5-phosphatase SHIP2. The substrate was preincubated with different concentrations of PCI as indicated (▶Figure 7) and the reaction was started by adding recombinant human SHIP2. After 1 h the reaction was stopped, and the released phosphate was measured. The results showed that low concentrations of PCI had a stimulatory effect on the activity of SHIP2 towards soluble dioleoyl-PtdIns(3,4,5)P_3 while high concentrations did not affect the removal of the 5-phosphate from the inositol headgroup (▶Figure 7). Furthermore A1AT or AT, two related serpins, did not show a modulatory effect on SHIP2 activity in the same experiment (not shown).

**Discussion**

We and others previously analysed the interaction of PCI with classical membrane phospholipids like PS, PE and PC (8, 9). To complete our understanding on lipids that might serve as additional non-protease ligands of PCI, we screened for new lipid-serpin interaction partners in this work. Surprisingly, the complexity of those interactions has exceeded expectations.

![Figure 5: Influence of monophosphorylated PIPs on the inhibition of APC by PCI. APC (1 nM) was incubated with different concentrations of human plasma PCI (pPCI, as indicated) for 60 min at 37°C in the absence or presence of different PtdInsP at the indicated concentrations. Heparin was used as a positive control at a concentration of 5 U/ml. Remaining APC activity was quantified using the chromogenic substrate S-2366 as described in Materials and methods. The activity of APC in the absence of PCI was set as 1. For each replication different preparations of human plasma PCI were used. Data represent mean values ± SEM, n = 3. All indicated concentrations are final concentrations.](https://example.com/image)
Interestingly the comparison of the results from the qualitative dot blots with those from the commercially available PIP-strips indicated that fatty acid composition and fatty acid oxidation is a crucial factor for the binding of PCI to DAG, PA and PS. Although no quantification of the dot blots in Figure 1 was performed, it is obvious that the presence of unsaturated fatty acids reduced the signal intensity for the binding of PCI to DAG and PA. In contrast to that observation the binding of PCI to PS using PIP-strips was weak compared to the binding of PCI to PS carrying palmitic acid at position sn-1 and arachidonic acid at position sn-2. So we concluded that for the binding of PCI to PS carrying palmitic acid at position sn-1 and arachidonic acid at position sn-2. We so we concluded that for the binding of PCI to PS carrying palmitic acid at position sn-1 and arachidonic acid at position sn-2. We so we concluded that for the binding of PCI to PS carrying palmitic acid at position sn-1 and arachidonic acid at position sn-2.

The inhibition of APC by PCI in the presence or absence of PIPs was used as a model system to investigate the potential effect of PIPs on protease inhibition. Interestingly, saturated monophosphorylated PIPs show a stimulatory heparin-like effect. Heparin and other effectors stimulate PCI’s inhibitory property towards different proteases by forming a bridged Michaelis-like complex (28). The ternary complex is strongly protease dependent and requires a flexible mode of heparin binding by PCI (29). In contrast to AT3, no ligand-dependent allosteric activation of PCI was observed so far. Therefore it is rather unlikely that a single phospholipid molecule is able to stimulate PCI.

So far only one docking model of PCI with unsaturated PE is available, showing that the lipid nicely fits into a binding pocket adjacent to helix H and helix D of the serpin (11). In addition D-myo-inositol 3,4,5,6-tetrakisphosphate was recently identified as...
Figure 7: SHIP2 activity in absence and presence of PCI. Dioleoyl-PtdIns(3,4,5)P$_3$ was preincubated for 10 min with different concentrations of PCI purified from human plasma (pPCI) as indicated. The reaction was started by adding recombinant human SHIP2 followed by incubation for 1 h at 37°C. Released free phosphate was detected using malachite green reagent. The samples were kept for 10 min at room temperature for colour development before measuring the absorbance at 620 nm. Phosphate release was quantified using a standard curve of K$_2$HPO$_4$ in assay buffer. **, p < 0.01 and *, p < 0.05 as determined using one-way ANOVA followed by Dunnett’s post test (n = 3). Data represent mean values ± SEM.

high-affinity ligand of AT3 interacting with the heparin-binding site (30). Further binding studies are needed to clarify whether PCI and other serpins possess additional binding pockets for the interaction with differently phosphorylated inositol head groups or other lipids.

How phospholipids are able to support ternary complex formation may be explained by the fact that they start to aggregate above the critical micelle concentration (CMC) resulting in the generation of charged surfaces. In that case the enhancement of serpin mediated protease inactivation by phospholipids should be dependent on the CMC. The concentration dependence of the stimulatory effect of PIPs on the inhibition of APC by PCI strengthens this assumption, since the amount of remaining free phospholipids in solution will always stay constant upon micelle formation. However, CMCs of most PIPs are unknown and can only be estimated (31). The CMC of unphosphorylated PtdIns from bovine liver containing predominantly stearic acid at position sn-1 and arachidonic acid at position sn-2 was determined to be ~ 16 µM (32). Addition of phosphate residues to the inositol headgroup will increase headgroup size and charge leading to an increase of the CMC. PtdIns(4,5)P$_2$ from porcine brain containing predominantly SA-PtdIns(4,5)P$_2$ exhibits a CMC of 30-40 µM (33). For our experiments we used monophosphorylated PIPs at 50 µM and 100 µM which should be far above the CMC and observed moderate modulation of PCI activity.

Interestingly, bispophosphorylated PIPs did not stimulate the inhibitory activity of PCI towards APC although binding of PCI to PtdIns(4,5)P$_2$ can be assessed as judged from native PAGE analysis and protein overlay assays. As mentioned above, ternary complex formation requires binding of both serpin and protease to the modulatory ligand. Thus, this finding might be explained by the lower affinity of PCI for higher phosphorylated PIPs as judged from binding assays. Secondly, the affinity of APC for PIPs has to be taken into account. However, understandably APC binding to PIPs has not been studied so far and therefore data on the interaction of APC with PIPs are not available.

In contrast to PIPs, the CMC of activated fatty acids is well studied and was found to be dependent on the acyl chain length. Saturated 16:0-CoA and 18:0-CoA exhibit CMCs of 42 µM and 7.5 µM, respectively (34). This might explain the huge differences in stimulation of APC inhibition by PCI, which we observe here. The reduced stimulatory effect of arachidonoyl-CoA compared to the saturated acyl-CoAs is not surprising since double bonds in the fatty acid chain lead to an increase of CMC compared to the corresponding saturated acyl chain (34).

From these results we can conclude that the stimulation of protease inhibition by phospholipids is dependent on the generation of charged surfaces.

Since PCI interacts with, and is stimulated by unsaturated negatively charged phospholipids (8) we hypothesise that the activation of AKT might be a consequence of direct interaction between PCI and PIPs. To our knowledge, this is the first time that the interaction of a serpin with PIPs is studied. As judged from protein lipid overlay assays, PCI shows a specific binding pattern towards different PIPs. The structurally comparable A1AT which shares 41.6% amino acid sequence identity with PCI (35) was also analysed in this assay, as was AT3. Although AT3 as well as A1AT bind to heparin (36, 37), those two serpins do not interact with PIPs in protein-lipid overlay assays. Consistent with previously published data on the binding of PCI to unsaturated PS, oxidised PS or oxidised PE (8), heparin slightly influenced the binding of PCI to PIPs indicating that the H-helix region of PCI might not only be involved in heparin binding but also contributes to PIP binding (not shown).

As mentioned above, some A-clade serpins seem to activate AKT via a PI3K-dependent pathway. Endothelial cells incubated with kallistatin (SerpinA4) for example show a 1.5-fold induction of AKT phosphorylation compared to control, which contributes to a protective effect against vascular damage and supports endothelial cell function (12). Vaspin (SerpinA12) on the other hand is an adipokokine which activates AKT in HAECs and prevents free fatty acid induced apoptosis in insulin-stimulated cells (13). Here we can demonstrate that addition of PCI from outside as well as intracellular PCI shows the ability to activate AKT, suggesting that the mechanism does not necessarily require interaction with cell surface receptors. However, the exact mechanism of AKT activation is not fully understood and the direct interaction of PCI with PIPs as well as new intracellular protein interaction partners might be possible explanations for this new and interesting function.

In addition, our group could identify JFC1 (synaptotagmin-like protein 1), as a new intracellular interaction partner of PCI (Sokolikova, unpublished data). JFC1 binds to 3'-phosphoinositides, is a target protein of protein kinase B (AKT) and its subcellular localisation seems to be dependent on its phosphorylation status (38, 39). By influencing AKT activation, PCI triggers PI3-kinase/AKT dependent JFC1 phosphorylation and might change the subcellular localisation status of both proteins. Therefore we think that PCI...
might influence a variety of intracellular processes related to the PI3-kinase/AKT pathway, and further studies are needed to elucidate the exact underlying mechanism. Secondly PCI was shown to interact with histone-lysine N-methyltransferase SUV420H1 (Sokolikova, unpublished data) suggesting a potential role of nuclear PCI in histone modification. This is not surprising since it was already shown that nuclear α1-antichymotrypsin (SerpinA3) strongly binds to chromatins and influences cell-cycle progression (40). Taken together those results are a promising starting point for future research on the potential intracellular and nuclear functions of PCI and other predominantly secreted serpins.

Unexpectedly, PCIs affinity for PIPs happens to be in the μM range and is ~500 fold lower compared to more abundant membrane lipids like PS, oxidised PS and oxidised PE (8). However the scansite prediction tool (41) scores a PtdIns(3,4,5)P3 pleckstrin homology (PH)-binding motif within the PCI sequence with a value of 0.701. Together with the multispecific binding pattern of PCI observed in protein overlay assays, these data are not surprising since 90% of the PH domain family members seem to bind PIPs very weakly and show a rather low specificity (42).

The interaction of intracellular serpins with phospholipids might influence intracellular lipid levels, the remodelling of cellular membranes and lipid signalling. Here we studied the influence of PCI on the activity of the PIP-dependent 5-phosphatase SHIP2. One of the rare examples that are known to enhance SHIP2 phosphatase activity is the adaptor protein with PH and SH2 domains (APS). APS specifically binds to the central region of SHIP2 and stimulates its activity in a concentration-dependent manner (43). Our data show that PCI stimulates SHIP2 activity by binding to the substrate PtdIns(3,4,5)P3. Interestingly a bell-shaped readout dependent on PCI concentration is observed indicating that the PCI:SHIP2 ratio influences the modulation of SHIP2 activity. When PCI is present in moderate excess (10-20 fold) over SHIP2, a stimulatory effect up to 30% is observed. Vast excess of PCI >60 fold over SHIP2 brings the activity back to control levels. One explanation for the stimulatory effect of PCI on SHIP2 activity in our assay might be a potential stabilising effect of the serpin on the soluble lipid substrate, making it easier for the phosphatase to attack the lipid headgroup. Higher PCI:SHIP2 ratios are not stimulating the phosphatase reaction any more and the activity goes back to baseline level.

This interesting and unexpected finding might be a new and yet unknown mechanism how cells could control PIP signalling by the uptake of PCI. Taken together our results indicate that the interaction of internalised PCI with PIPs might affect intracellular lipid signalling and contribute to the regulation of important cellular functions.

Conflict of interest
None declared.

References