

Type II phosphoinositide 5-phosphatases have unique sensitivities towards fatty acid composition and head group phosphorylation

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Abstract The catalytic properties of the type II phosphoinositide 5-phosphatases of Lowe's oculocerebrorenal syndrome, INPP5B, Synaptojanin1, Synaptojanin2 and SKIP were analysed with respect to their substrate specificity and enzymological properties. Our data reveal that all phosphatases have unique substrate specificities as judged by their corresponding K_M and V_{Max} values. They also possessed an exclusive sensitivity towards fatty acid composition, head group phosphorylation and micellar presentation. Thus, the biological function of these enzymes will not just be determined by their corresponding regulatory domains, but will be distinctly influenced by their catalytic properties as well. This suggests that the phosphatase domains fulfil a unique catalytic function that cannot be fully compensated by other phosphatases.

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1. Introduction

Phosphoinositide (PI) phosphatases can be classified according to their sequence homology in two categories; the CX5R phosphatases, which share homology to and are part of the large tyrosine phosphatase family, and the type II phosphatases that form a distinct small family on their own [1]. Based on their domain structure, type II phosphatases containing an N-terminal SAC1, an C-terminal BCR-GAP and an N-terminal SH2 or proline rich domain have been classified as SAC domain

containing inositol phosphatases (SCIPs), GAP domain containing inositol phosphatases (GIPs), SH2 domain containing inositol phosphatases or proline-rich domain containing inositol phosphate phosphatases, respectively [2]. However, the recently discovered PI phosphatase skeletal muscle and kidney enriched inositol phosphatase (SKIP) has no obvious domains apart from the type II phosphatase homology domain and a small membrane ruffle targeting domain [3] and thus has to be classified as a uniquely simple member of the type II phosphatase family [4].

Many members of the type II phosphatase family are important mediators of cellular signalling or membrane traffic and play an important role in human health [1,5]. In particular, the gene product responsible for Lowe's oculocerebrorenal syndrome (OCRL), a member of the GIP phosphatase class, comes to mind [6]. The disease is caused by the loss of the catalytic activity of the OCRL phosphatase, which cannot be compensated by the other known member of the GIP class, the INPP5B phosphatase in humans, although they share high homology in the phosphatase and BCR-GAP domain [7]. The cellular function of OCRL and other type II phosphatases is determined by the actual catalytic properties of the type II phosphatase domain and by the localisation of the enzyme [8,9], the latter being determined by the domains that are able to interact with proteins and/or compartments such as the SH2, BCR-GAP and proline rich domains [1]. Many of these type II phosphatases are subject to alternative splicing in the regions of these targeting domains, thus creating differently localised phosphatases with similar substrate specificity. For example, synaptojanin2 is spliced in its C-terminal proline rich domain, resulting in six different splice forms with unique cellular localisation, whereas synaptojanin1 splicing in the proline rich domain results in differential developmental expression levels [10,11]. While there is some evidence concerning the importance of the targeting of phosphatases towards their distinctive compartments [3,9,12,13], the substrate specificity and catalytic properties of the corresponding phosphatase domains are still ill defined. In particular, the enzymological properties towards some inositol lipid substrates of these phosphatases are available for a few of these enzymes [4,14,15]. However, due to variations in the assay conditions employed the available data are not suited for a comparative analysis.

Here, we will describe the comparative determination of the enzymological properties of the phosphatase domains of the

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Abbreviations: GIP, GAP domain containing inositol phosphatase; OCRL, Lowe's oculocerebrorenal syndrome; OG, octylglycoside; PI, phosphoinositide; PtdIns, phosphatidylinositol; PtdIns-3,4,5-P3dC16, L- α -D-myo-phosphatidylinositol 3,4,5-triphosphate 3-O-phospho linked, D(+)-sn-1,2-di-O-hexadecanoylglycerol; SCIP, SAC domain containing inositol phosphatase; SKIP, skeletal muscle and kidney enriched inositol phosphatase

two SCIPs, synaptojanin1 and 2, the two GIPs, OCRL and INPP5B, and the full length SKIP phosphatase. All tested phosphatases showed an ability to hydrolyse phosphatidylinositol 4,5 bisphosphate (PtdIns-4,5-P2), but differed substantially in their ability to employ PtdIns-3,4,5-P3 or Ins-1,4,5-P3. Differential sensitivities towards the head group and fatty acid composition of the lipid substrates were observed suggesting that the phosphatase domains fulfil a unique catalytic function that cannot be fully compensated by other phosphatases.

2. Methods

2.1. Bacterial expression of PI 5-phosphatase domains

The boundaries of the phosphatase domains of synaptojanin1 (rat; S470-R962) and synaptojanin2 (human; D474-R959), OCRL (human; V202-E618) and INPP5B (mouse; A305-A721) were modelled on the full length SKIP phosphatase (human), which roughly resembles the boundaries used recently for obtaining the crystal structure of this domain [16]. The phosphatase domain constructs of the five different phosphoinositide 5-phosphatases were cloned into the pGEX-4T2 expression vector (Pharmacia). These plasmids were transfected into a DH5 α strain of *Escherichia coli* and the phosphoinositide 5-phosphatases were expressed while bacteria were induced with 100 μ M isopropyl- β -D-thiogalactopyranoside at 18 °C overnight. The glutathione *S*-transferase-fusion proteins were affinity-purified using glutathione sepharose 4B (Pharmacia) and eluted with 20 mM reduced glutathione and 500 mM NaCl, pH 8. Several mg of proteins of sufficient purity were regularly obtained using the described method for expression and purification (data not shown).

2.2. Malachite green phosphate release assay

The enzyme activity of the PI 5-phosphatases was measured with a well-established phosphate release assay using an acidic malachite green dye [17]. These assays were performed in the presence of water-soluble inositol phosphates or phosphoinositides, respectively. Lipid substrates were dried down, resuspended in 1% octylglucoside (OG), which had the least interference with the colorimetric phosphate release determination, and sonicated for 10 min. The buffer conditions for all experiments were as followed: 200 mM Tris, pH 7.4, 50 ng/ μ l BSA. The phosphatase reaction was initiated by adding the enzyme to the substrates and samples were incubated at 30 °C. In order to stop the enzyme reaction, 0.7 volume of the acidic dye solution (2.3 mg/ml malachite green in 3.6 M HCl and 17 mM ammonium molybdate) was added. The mixture was allowed to develop for 20 min and OD₆₂₅ was measured.

2.3. Testing substrate specificity

To analyse the substrate specificity of all five phosphoinositide 5-phosphatases, we employed the above described phosphate release assay using the following substrates; PtdIns-4,5-P2nat (Lipid Products, UK), PtdIns-4,5-P2dC16, L- α -D-myo-phosphatidylinositol 3,4,5-triphosphate 3-*O*-phospho linked, D(+)-sn-1,2-di-*O*-hexadecanoylglycerol (PtdIns-3,4,5-P3dC16), PtdIns-3,5-P2dC16, PtdIns-4-PdC16, PtdIns-5-PdC16, Ins-1,5-P2, Ins-1,4,5-P3 and Ins-1,3,4,5-P4 (Cell Signals). In order to achieve an OD₆₂₅ of approximately 0.5 for all 5-phosphatases using 50 μ M PtdIns-4,5-P2dC16 different enzyme concentrations were employed, which allowed us to compare the different recombinant 5-phosphatases to each other as a percentage of the PtdIns-4,5-P2 activity. All experiments were carried out in the presence of 50 μ M substrate (in the presence of 0.25% octylglucoside) at 30 °C for 30 min and were repeated twice.

2.4. K_M – V_{Max} analysis

To analyse the enzymatic properties of the PI 5-phosphatases, we established K_M and V_{Max} values for the natural and the synthetic PtdIns-4,5-P2 and PtdIns-3,4,5-P3dC16 for all five proteins. The water soluble Ins-1,4,5-P3 was only examined for OCRL, INPP5B and synaptojanin1. SKIP and synaptojanin2 lack the ability to hydrolyse this inositol. Furthermore, Ins-1,4,5-P3 was tested in the presence and absence of octylglucoside. We detected that the detergent changed the enzymatic properties, which made this additional approach necessary.

Ascending concentrations up to 300 μ M of sonicated lipid and inositol phosphates, respectively, were pre-incubated at 30 °C and the phosphate release assay was initiated by adding the PI 5-phosphatase. In order to achieve linear assays, the conditions for these K_M/V_{Max} studies were optimised in respect to time and enzyme concentration. OD₆₂₅ values were plotted against substrate concentration and K_M and V_{Max} values were calculated using GraphPad Prism software.

3. Results

3.1. Substrate specificity of the type II phosphatase domains

In order to compare the substrate specificity of the different phosphatases, it was important to assess first the substrates that have significant turnover. The catalytic domains of these phosphatases, modelled on the boundaries of the shortest member of the type II 5-phosphatase family (SKIP) [4], were therefore, tested for their ability to employ the lipids in a mixed micelle assay. As shown in Fig. 1, all tested phosphatase domains were able to hydrolyse efficiently PtdIns-4,5-P2 and to a lesser extent PtdIns-3,4,5-P3, whereas other inositol lipids and phosphates are hydrolysed sufficiently only by some of the tested phosphatases only. All tested phosphatase domains were unable to efficiently hydrolyse PtdIns-5-P or its corresponding head group Ins-1,5-P2, suggesting a preference for bis-phosphorylated inositol lipids. Indeed, PtdIns-4,5-P2 (natural and/or synthetic) seems to be the preferred substrate for all tested type II phosphatases, since hydrolysis of any other phosphoinositide lipid was in comparison with either of similar or weaker strength. Interestingly, PtdIns-3,5-P2 is not a good substrate to any phosphatase except OCRL, whereas synaptojanin2 and SKIP did not show any significant turnover of the soluble inositol phosphates Ins-1,4,5-P3 and Ins-1,3,4,5-P4. However, synaptojanin1 did possess sufficient activity towards Ins-1,4,5-P3, but had much lower reactivity when exposed to Ins-1,3,4,5-P4.

3.2. Sensitivity of the type II phosphatases towards head group phosphorylation, fatty acid composition and micelle presentation

The substrate specificity assessment revealed that the closely related phosphatase domains did have significant turnover towards PtdIns-4,5-P2 and PtdIns-3,4,5-P3 as well as Ins-1,4,5-P3 (Fig. 1). The enzymological parameters K_M (substrate affinity) and V_{Max} (catalytic turnover) for these substrates were therefore assessed (Table 1). Given that these phosphatases have not been characterised in a comparative fashion with respect to these parameters before, it is reassuring that the observed K_M values for PtdIns-4,5-P2 are roughly in agreement with earlier investigations focussing on purified synaptojanin1 [15] or recombinant SKIP [4], respectively. Furthermore, the K_M values for Ins-1,4,5-P3 for INPP5B was also closely resembling earlier observations [14], thus validating our enzymological characterisation and design of phosphatase domains. The V_{Max} values are not easily compared to any published data, since these values rely ultimately on the purity (specific activity) of the enzyme preparation, which will inevitably vary. However, while it is meaningless to compare the turnover (V_{Max}) of the different phosphatase domains for a given substrate, it is possible to compare the relative turnover (V_{Max} as percentage of the PtdIns-4,5-P2dC16 activity) of the different substrates for a given phosphatase domain (see Table 1).

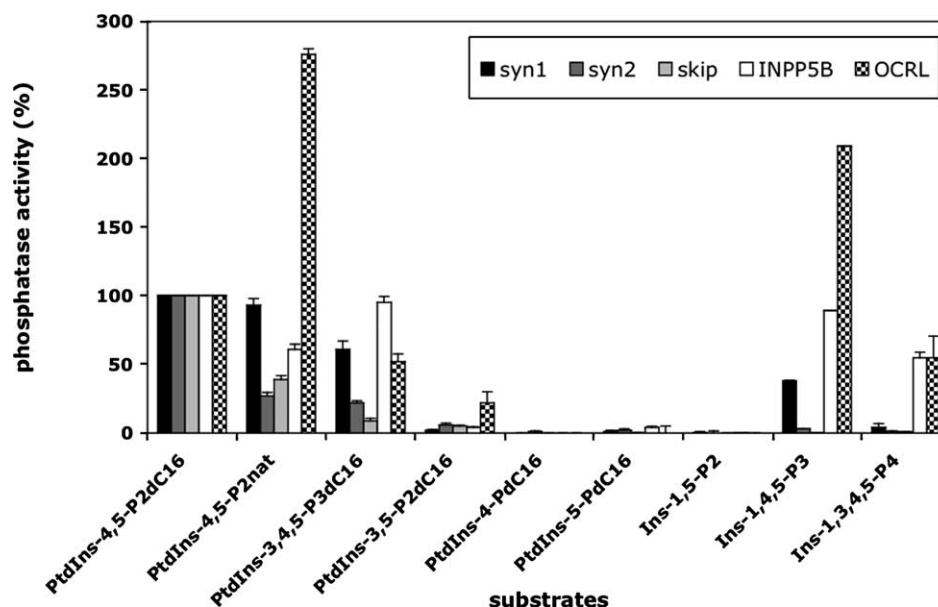


Fig. 1. Substrate specificity of recombinant catalytic domains of the PI 5-phosphatase. Phosphate release assays were carried out using 50 μ M of the following substrates: PtdIns-4,5-P2nat, PtdIns-4,5-P2dC16, PtdIns-3,4,5-P3dC16, PtdIns-3,5-P2dC16, PtdIns-4-Pnat, PtdIns-4-PdC16, PtdIns-5-PdC16, Ins-1,5-P2, Ins-1,3,5-P3 and Ins-1,3,4,5-P4. Enzyme reactions were initiated by adding the protein and incubated at 30 $^{\circ}$ C for 30 min. The phosphatase activity is presented as a percentage of the observed PtdIns-4,5-P2 activity employing a synthetic dihexadecanoyl PtdIns-4,5-P2 (PtdIns-4,5-P2dC16). All other synthetic inositol lipids employed contain the same fatty acid composition in order to focus on the influence of the head group on the substrate specificity. However, for comparison a PtdIns-4,5-P2 (PtdIns-4,5-P2natural), which had been purified from mammalian brain tissue and thus contained an fatty acid composition resembling the natural in vivo environment (i.e., unsaturated fatty acids), was employed as well. PtdIns-4-PdC16 was used as a negative control. The results were presented as the means \pm S.E. of triplicate determinations.

The comparative analysis of the K_M and V_{Max} values led to some conclusion with respect to the sensitivity of each phosphatase domain towards the head group, fatty acid composition and micellar presentation. As summarised in Table 2 each tested 5-phosphatase had quite distinct properties, which did not correlate with the shared primary sequence homology. All tested phosphatase domains showed a unique response towards (i) fatty acid composition (PtdIns-4,5-P2dC16 vs PtdIns-4,5-P2natural), (ii) head group phosphorylation (PtdIns-4,5-P2dC16 vs PtdIns-3,4,5-P3dC16) and (iii) micellar presentation (Ins-1,4,5-P3+OG vs Ins-1,4,5-P3-OG). With regard to the latter, only the two GIPs and synaptojanin1 were able to process significant amounts of Ins-1,4,5-P3, preferably without detergent micelles being present, but each phosphatase responded to the micellar presentation in a different fashion (OCRL, change of K_M and V_{Max} ; INPP5B, change of K_M only; synaptojanin1, change of V_{Max} only). Similarly, the fatty acid composition of the lipid substrate (presented as a mixed micelle) is sensed by each phosphatase in a different fashion (OCRL, moderate change in V_{Max} only; INPP5B, moderate change in K_M and V_{Max} ; Synaptojanin1 (Syn1), moderate change in K_M but a very strong change in V_{Max} ; Synaptojanin2 (Syn2), no change in either K_M or V_{Max} ; SKIP, substantial change in K_M and V_{Max}). This unique sensing of the phosphatase domains is also detectable for the head group phosphorylation, a feature that should result in changes in the affinity (K_M) of the phosphatase towards its substrate. Indeed, as expected all tested phosphatases responded with a significant change in the K_M values. However, some phosphatases (INPP5B, synaptojanin1 and SKIP) also responded by changing the V_{Max} values. In particular, the SKIP phosphatase

had a 50-fold lower V_{Max} for PtdIns-3,4,5-P3 as compared to PtdIns-4,5-P2, effectively overriding the 5-fold increase in affinity (K_M) and thus making this enzyme an effective and specific PtdIns-4,5-P2 5-phosphatase. However, it cannot be excluded given SKIP decreases insulin-induced PtdIns(3,4,5)P₃ intracellular levels and Akt phosphorylation, that upon plasma membrane recruitment SKIP is a more efficient PtdIns(3,4,5)P₃ 5-phosphatase in intact cells [21].

4. Discussion

The here observed differences in the enzymological properties and sensitivities of the type II phosphatases (GIPs, SCIPs and SKIP) demonstrated the uniqueness of these enzymes, which will have a bearing on their biological function. For example, the OCRL phosphatase is supposed to be a PtdIns-4,5-P2 phosphatase, since the absence of its activity results in higher PtdIns-4,5-P2 level in vivo [18]. However, knockouts in mice failed to create a “Lowe syndrome” phenotype, and it has been speculated that the other member of the GIP family, INPP5B, may be able to rescue any loss of function due to OCRL [7]. Our data presented here (Table 2) clearly show that these two GIP phosphatases are distinct in their catalytic properties and sensitivities. OCRL is a PtdIns-4,5-P2 5-phosphatase that senses the fatty acid composition as demonstrated by a corresponding change in V_{Max} . INPP5B on the other hand is not sensing the fatty acid composition, since only moderate or insignificant changes in K_M and V_{Max} are recorded. Both GIPs prefer PtdIns-4,5-P2 to PtdIns-3,4,5-P3, albeit due to different mechanisms. OCRL has a better affinity for

Table 1
Enzymological properties of the type II phosphatases

	K_M (μ M)	V_{Max} (U/l)	V_{Max} (%)	K_M/V_{Max}
OCRL				
PtdIns-4,5-P2dC16	22 \pm 8	45 \pm 20	100	0.22
PtdIns-4,5-P2natural	23 \pm 4	167 \pm 20	371	0.06
PtdIns-3,4,5-P3dC16	139 \pm 62	46 \pm 20	102	1.36
Ins-1,4,5-P3+OG	143 \pm 59	13 \pm 6	29	4.93
Ins-1,4,5-P3-OG	34 \pm 9	88 \pm 12	196	0.17
INPP5B				
PtdIns-4,5-P2dC16	135 \pm 61	91 \pm 24	100	1.35
PtdIns-4,5-P2natural	48 \pm 15	41 \pm 13	45	1.06
PtdIns-3,4,5-P3dC16	44 \pm 13	12 \pm 4	13	3.38
Ins-1,4,5-P3+OG	194 \pm 60	56 \pm 17	61	3.18
Ins-1,4,5-P3-OG	55 \pm 22	58 \pm 13	64	0.86
Syn1				
PtdIns-4,5-P2dC16	27 \pm 7	31 \pm 3	100	0.27
PtdIns-4,5-P2natural	100 \pm 21	401 \pm 34	1294	0.08
PtdIns-3,4,5-P3dC16	123 \pm 28	83 \pm 40	267	0.46
Ins-1,4,5-P3+OG	157 \pm 43	9 \pm 5	29	5.4
Ins-1,4,5-P3-OG	141 \pm 30	43 \pm 5	139	1.01
Syn2				
PtdIns-4,5-P2dC16	54 \pm 11	15 \pm 9	100	0.54
PtdIns-4,5-P2natural	90 \pm 34	34 \pm 16	226	0.4
PtdIns-3,4,5-P3dC16	113 \pm 36	21 \pm 4	140	0.81
SKIP				
PtdIns-4,5-P2dC16	107 \pm 30	298 \pm 50	100	1.07
PtdIns-4,5-P2natural	23 \pm 11	55 \pm 11	18	1.28
PtdIns-3,4,5-P3dC16	21 \pm 10	5 \pm 2	2	10.5

Purified recombinant type II 5-phosphatases (phosphatase domains only) were analysed with respect to their corresponding K_M and V_{Max} values using the indicated substrates as described in Section 2. For comparison the V_{Max} value (given in U/l; U = μ mol/min) is also shown as a percentage of the PtdIns-4,5-P2dC16 value, the substrate all tested phosphatases preferred. The ratio K_M/V_{Max} is shown as an indicator of the substrate preference (low values indicating a good substrate).

PtdIns-4,5-P2, while INPP5B has a better turnover for this lipid substrate. The two enzymes are hydrolysing the soluble inositol trisphosphate (in the presence of OG), but again show quite distinct characteristics. OCRL has a better affinity towards PtdIns-4,5-P2 than to Ins-1,4,5-P3, whereas INPP5B has similar affinities for both substrates. Although it cannot be excluded that other domains present in the full length phosphatases influence the observed substrate specificity, it is more likely that these domains modulate the biological function, rather than the catalytic activity, due to targeting to different

compartments (membranes) and/or protein complexes (cytoskeleton). Therefore, the apparent functional overlap in vivo between mouse *Ocrl1* and *Inpp5b* [7] must be a reflection of a complex crosstalk of the metabolic pathways in which these two enzymes participate rather than a direct compensation for the loss of phosphatase activity in OCRL patients by INPP5B. The ability of OCRL to hydrolyse PtdIns-3,5-P2 is also of interest in that this phosphoinositide is known to be involved in golgi trafficking where the OCRL enzyme is located [19].

In a similar fashion, the two members of the SCIP family reveal distinct enzymological properties and sensitivities. Firstly, synaptojanin2 does not work well on soluble inositol phosphates. In contrast, synaptojanin1, like OCRL, is able to use Ins-1,4,5-P3, although the natural PtdIns-4,5-P2 is by far the preferred substrate (Table 2). This ability to work on the Ins-1,4,5-P3 does allow synaptojanin1 to exert control on the corresponding calcium rises, which has been recently demonstrated in chinese hamster ovary cells [20]. However, unlike OCRL, synaptojanin1 is much more accommodating towards PtdIns-3,4,5-P3, since the difference in the K_M/V_{Max} values is less severe. This ability to hydrolyse PtdIns-3,4,5-P3 efficiently is in agreement with earlier observations [21]. Synaptojanin2 is overall characterised by an indifference towards head group phosphorylation and fatty acid composition (moderate change in K_M/V_{Max} values) suggesting that synaptojanin2 is a 5-phosphatase for higher phosphorylated inositol lipids only.

Finally, the substrate specificity of SKIP is quite unique, since it is almost exclusively hydrolysing PtdIns-4,5-P2. SKIP does not have any significant activity towards the soluble inositol phosphates and is characterised by a distinctly low turnover for PtdIns-3,4,5-P3, a recently proposed substrate in vivo [22]. However, the unique exclusiveness of SKIP is similar to that of the *S. cerevisiae* gene product *INP54* [23] suggesting that both enzymes might govern similar cellular responses. In summary, our data reveal that the closely related type II phosphatases are very unique in their catalytic properties, implying that their corresponding biological function is not only determined by their regulatory domains and thus localisation, but their intrinsic catalytic properties. Our data reveal as well that these phosphatases possess unique sensitivities towards fatty acid and head group composition of the lipid substrate.

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Table 2
Sensitivities of the type II phosphatases

Enzyme	Fatty acid		Head group		Micelle		Best substrate
	K_M	V_{Max}	K_M	V_{Max}	K_M	V_{Max}	
OCRL	NS	371	632	NS	421	15	PtdIns-4,5-P2nat
INPP5B	36	45	33	13	353	NS	PtdIns-4,5-P2
Syn1	370	1294	456	267	NS	21	PtdIns-4,5-P2nat
Syn2	NS	NS	209	NS	NA		PtdIns-4,5-P2
SKIP	21	18	20	2	NA		PtdIns-4,5-P2dC16

The data presented in Table 1 were analysed with respect to sensitivity towards fatty acid composition (PtdIns-4,5-P2dC16 vs. PtdIns-4,5-P2natural), head group phosphorylation (PtdIns-4,5-P2dC16 vs. PtdIns-3,4,5-P3dC16) and micelle presentation (Ins-1,4,5-P3+OG vs Ins-1,4,5-P3-OG). The table indicates the significant changes of the K_M and/or V_{Max} values as a percentage of the corresponding values obtained for PtdIns-4,5-P2dC16 or Ins-1,4,5-P3-OG (NS = no significant change). As synaptojanin2 and SKIP do not possess significant activities towards Ins-1,4,5-P3 no conclusion concerning the sensitivity towards micellar presentation could be drawn (NA = not applicable). The determination of the best substrate was based on significant changes in the K_M/V_{Max} ratio ("PtdIns-4,5-P2" indicating similar preferences for both tested PtdIns-4,5-P2 lipids).

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