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Specificity of the purified inositol (1,3,4,5) tetrakisphosphate-binding protein from porcine platelets

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Abstract The specificity of the inositol 1,3,4,5-tetrakisphosphate binding protein purified from porcine platelets [Cullen et al. (1995) Biochem. J. 305, 139–143] was examined using all the isomers of *myo*-inositol tetrakisphosphate. From the relative potencies of these compounds it appears that phosphorylation of the 1, 3 and 5 positions is essential for high affinity binding, that there is some tolerance of phosphorylation of the 6-hydroxyl, but none of a phosphate in the 2-position, and that phosphorylation of the 4-hydroxyl has very little influence. The binding of Ins(1,3,4,5)P_4 was not appreciably altered by physiological Mg^{2+} concentrations, and the pH dependence of binding under physiological conditions showed a decline from pH 5.5 to pH 9.0.

Key words: Inositol; Inositol tetrakisphosphate; Binding protein; Platelet; Procine; Receptor

1. Introduction

The issue of whether or not Ins(1,3,4,5)P_4 is a second messenger, and if so what its function(s) may be, is currently still controversial [1,2]. One route to resolving the issue is to identify the molecular nature of putative Ins(1,3,4,5)P_4 receptors [3], and to that end we have recently purified to homogeneity from porcine platelets an Ins(1,3,4,5)P_4-binding protein which shows a high affinity and specificity for Ins(1,3,4,5)P_4 [4]. In our original study of the specificity of this protein, we investigated the efficacy of a number of other inositol phosphates, including three InsP_4's (the 1,3,4,5; 3,4,5,6; and 1,3,5,6 (=L-1,3,4,5)) isomers, but this gives only an incomplete picture of the specificity of this protein. As yet we have only a very vague picture of the requirements of this protein with respect to which hydroxyls on the inositol ring must be phosphorylated for optimal binding (compare with our much more comprehensive knowledge of the Ins(1,4,5)P_3 receptor). Two of us (S-K.C. and Y-T.C.) have recently synthesised all the tetrakisphosphate isomers of *myo*-inositol [5]. The twelve asymmetric isomers are made as six racemic pairs and so cannot be tested individually, but in the present context (displacement of [^{32}P]Ins(1,3,4,5)P_4 from the protein) this does not matter, because each pair will be dominated by the most potent enantiomer; moreover, as discussed below, other data with selected enantiomerically pure compounds resolves any ambiguity. Thus using these samples enables us to get an essentially complete picture of the isomeric specificity of the binding site on the pure protein.

We also here report a few other properties of this protein. In particular, the use of a pure protein, now devoid of any Ins(1,3,4,5)P_4 phosphatase activity, enables us for the first time to study Ins(1,3,4,5)P_4 binding under near physiological conditions, with Mg^{2+} present.

2. Materials and methods

The Ins(1,3,4,5)P_4 binding protein and radioligand (carrier-free [5-^{32}P]Ins(1,3,4,5)P_4) were prepared exactly as in [4] and binding assays were exactly as in that paper.

The nine *myo*-inositol tetrakisphosphates (six racemic pairs and three *meso*-isomers) were synthesised as in Chung and Chang [5]. In brief, inositol 1,4 bisbenzoate was randomised by base-catalyst isomerisation, and the nine resulting inositol bisbenzoate isomers were purified by hplc, and identified by ^{1}H and ^{13}C NMR including H-H COSY. Each of these was then phosphorylated by phosphitylation followed by oxidation, and de-protected by successive treatments with trimethylsilyl bromide and KOH.

3. Results and discussion

3.1. Binding specificity

Our previous studies [4] on the purified Ins(1,3,4,5)P_4-binding protein from porcine platelets yielded a K_d of 6.3 ± 0.4 nM for Ins(1,3,4,5)P_4 and K_ds of 85.0 ± 4.1 nM, 800.0 ± 20.2 nM, 65.6 ± 2.6 nM, > 10 μM, 793.3 ± 55.6 nM, and 85.0 ± 5.9 nM for Ins(1,3,4,5,6)P_5, InsP_6, Gro*P*Ins(3,4,5)P_3, Ins(1,4,5)P_3, Ins(3,4,5,6)P_4 and L-Ins(1,3,4,5)P_4 (=Ins(1,3,5,6)P_4), respectively.

As a first stage in investigating the efficacy of all the synthetic InsP_4 isomers, we conducted a preliminary screen using a partially purified InsP_4-binding protein fraction (yields of pure protein are sufficiently low that quantifying so many different compounds would require a major investment of material). We used the binding peak from the heparin Hi-Trap column [4] which, like crude solubilised platelet membranes, shows an apparent K_d of 56.5 ± 1.5 nM for Ins(1,3,4,5)P_4 and yields a curved Scatchard transformation which can be fitted to two sites ($P = 0.03$ compared with a one site fit) having K_ds of 26.2 ± 19.6 nM and 653.6 ± 440.0 nM (results not shown, compare with [4]).

The K_ds of the nine InsP_4 preparations are given in Table 1, and three things are readily apparent from these data. Firstly, D/L Ins(1,3,4,5)P_4 is half as potent as D-Ins(1,3,4,5)P_4, consistent with L-Ins(1,3,4,5)P_4 being weaker than the D-isomer [4]. Secondly, any InsP_4 with a phosphate in the 2-position is essentially inactive. As this is only a partially purified fraction with two

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Abbreviations: All inositol phosphate isomers are given in *D*-numbering unless otherwise stated.

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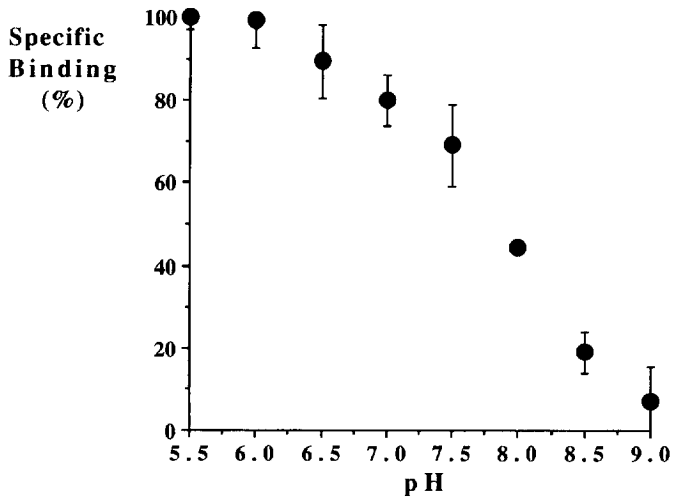

Fig. 1. Effect of pH on Ins(1,3,4,5)P_4 binding to the purified porcine platelet Ins(1,3,4,5)P_4-binding protein. Assays were performed as described in section 2 with buffers based on 100 mM KCl, 20 mM NaCl, 1 mM EDTA, 0.1% (w/v) BSA and either 10 mM MES, 10 mM HEPES and 25 mM Tris for pH's 5.5–6.5, 7.0–7.5 and 8.0–9.0, respectively. Non-specific binding was determined in the presence of 10 μM Ins(1,3,4,5)P_4.

apparent affinities, we cannot give precise numbers for what the potency of these isomers might be on the pure, high-affinity protein. Since all these isomers are so weak in this assay we can conclude that neither binding site can tolerate a phosphate in the 2-position in an $InsP_4$, and we have therefore not explored them further.

The third observation that stems from Table 1 is that D/L Ins(1,4,5,6)P_4 is the only other sample that shows any significant (i.e. less than two orders of magnitude weaker than Ins(1,3,4,5)P_4) ability to displace Ins(1,3,4,5)P_4. We therefore studied D/L Ins(1,4,5,6)P_4 further using the purified protein for which we already have K_ds of 6.3 ± 0.4 nM and 793.3 ± 55.6 nM for Ins(1,3,4,5)P_4 and Ins(3,4,5,6)P_4 (= L-Ins(1,4,5,6)P_4), respectively [4]. In this set of experiments D/L Ins(1,4,5,6)P_4 showed a K_d of 390.0 ± 48.9 nM compared with 6.4 ± 0.2 nM for Ins(1,3,4,5)P_4, suggesting that D-Ins(1,4,5,6)P_4 is probably slightly more potent than L-Ins(1,4,5,6)P_4, but that both are more than 50-fold weaker on the pure protein than Ins(1,3,4,5)P_4.

We can therefore now begin to suggest some criteria which can be regarded as being required for high affinity binding to this protein. As mentioned above, a 2-phosphate cannot be tolerated (see also the low affinity of $InsP_6$ [4]). There is some tolerance for a 6-phosphate (Ins(1,3,4,5,6)P_5 vs. Ins(1,3,4,5)P_4), and a 1-phosphate considerably strengthens binding (Ins(3,4,5,6)P_4 vs. Ins(1,3,4,5,6)P_5 [4], and see also Ins(3,4,5)P_3 vs. Ins(1,3,4,5)P_4 in [6]); there is some steric hindrance caused by a glycerol moiety on this phosphate (Gro*P*Ins(3,4,5)P_3 vs. Ins(1,3,4,5)P_4 [4]). A 3-phosphate is essential (Ins(1,4,5)P_3 vs. Ins(1,3,4,5)P_4), although if a 6-phosphate is added to Ins(1,4,5)P_3 (to give Ins(1,4,5,6)P_4), that can exert a small effect to increase affinity. A 5-phosphate is essential for binding (Ins(1,3,4,6)P_4 vs. Ins(1,3,4,5,6)P_5, and see also the lack of binding of Ins(1,3,4)P_3 in [6] and in many other studies on what are presumably similar binding sites, summarised in [1]). Phosphorylation in the 4-position makes very little difference (Ins(1,3,5,6)P_4 (= L-Ins(1,3,4,5)P_4) vs. Ins(1,3,4,5,6)P_5 [4]). Thus we can suggest that the binding site on this protein recognises primarily the 1, 3 and 5 phosphates, with no tolerance for a 2-phosphate, some tolerance of a 6-phosphate, and with the 4-phosphate having little influence.

3.2. *Effect of Mg^{2+}*

We have earlier emphasised how Ins(1,3,4,5)P_4 binding sites on membranes can be used to assay the mass of Ins(1,3,4,5)P_4 in unfractionated tissue extracts under both artificial [7,8] and nearer physiological [9] conditions. The latter study [9] was inevitably compromised, as are all such studies, by the omission of Mg^{2+} from and inclusion of EDTA in the binding assay –essential to avoid degradation of the ligand by Mg^{2+}-dependent phosphatases. It is entirely possible that Mg^{2+} might alter significantly the affinity or specificity of Ins(1,3,4,5)P_4 binding, and thus would alter our interpretation of these studies namely, that Ins(1,3,4,5)P_4 is the most likely natural ligand for these binding sites.

The use of a pure protein enabled us to check this, and so we measured the affinity of Ins(1,3,4,5)P_4, Ins(1,3,4,5,6)P_5, $InsP_6$ and Ins(1,4,5)P_3 with 2 mM Mg^{2+} present. There was a clear increase in non-specific binding (from about 10% to 30%) but specific binding was not altered (data not shown), and we could not detect any significant change in the apparent affinity of any of the inositol phosphates tested.

3.3. *Effect of pH*

We have shown before [9] that effects of pH on Ins(1,3,4,5)P_4 binding sites vary somewhat between tissues, and are also changed by the presence of 50 mM inorganic phosphate (or, put the way that we described it [9], the effect of inorganic phosphate is different at pH 5.5 vs. pH 7.0). We therefore examined (in the absence of inorganic phosphate) the effect of pH on the purified putative Ins(1,3,4,5)P_4 receptor, partly to document the behaviour of this particular non-neuronal protein, but also to compare with the behaviour of three neuronal Ins(1,3,4,5)P_4-specific binding proteins studied by Theibert et al. [10] using an $InsP_4$ affinity probe. Fig. 1 shows that at a single Ins(1,3,4,5)P_4 concentration, specific binding decreased steadily from pH 5.5 to pH 9.0. In this it most closely resembles the p84 of Theibert et al. [10] and is markedly different from the p182 and p174 (and also the $InsP_6$-binding protein, which the pharmacology of our present protein rules out as being the same). So, if any of these three proteins is the neuronal homologue of our platelet protein, on this criterion alone, the p84 is the most likely.

Table 1
Efficacy of synthetic $InsP_4$ isomers using a partially purified porcine platelet Ins(1,3,4,5)P_4-binding protein

Inositol phosphate (D/L isomer)	IC_{50} (μM)
Ins(1,2,4,5)P_4	8.1 ± 0.1
Ins(1,2,4,6)P_4	6.3 ± 0.1
Ins(1,2,3,5)P_4	> 10
Ins(1,3,4,6)P_4	8.2 ± 0.4
Ins(2,4,5,6)P_4	5.8 ± 0.1
Ins(1,3,4,5)P_4	0.098 ± 0.01
Ins(1,2,5,6)P_4	≥ 10
Ins(1,2,3,4)P_4	7.7 ± 0.1
Ins(1,4,5,6)P_4	1.7 ± 0.2
D-Ins(1,3,4,5)P_4	0.056 ± 0.001

Assays were performed at pH 7.0 as described in section 2.

4. Conclusion

Overall we can conclude that a more detailed study of the properties of the purified platelet Ins(1,3,4,5)P_4-binding protein has emphasised its specificity under physiological conditions, and so has increased rather than decreased the strength of our interpretation [3,4,9] that this protein is a reasonable candidate to be a physiological receptor for Ins(1,3,4,5)P_4. As discussed in [4], although the current data support Ins(1,3,4,5)P_4 as the most likely ligand, we cannot yet rule out PtdIns(3,4,5)P_3 as an alternative physiological target for this protein, and the high specificity shown here for what is the head-group of that lipid is consistent also with that possibility. Resolving these issues will clearly require further study.

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