Synthetic inositol 1,3,4,5-tetrakisphosphate analogues

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Inositol 1,3,4,5-tetrakisphosphate [$\text{Ins}(1,3,4,5)\text{P}_4$] analogues were synthesized and their effects on [^3H]Ins(1,3,4,5) P_4 5-phosphatase, [^3H]Ins(1,3,4,5) P_4 3-phosphatase and [^3H]inositol 1,4,5-trisphosphate [^3H]Ins(1,4,5) P_3 5-phosphatase activities were examined. The Ins(1,3,4,5) P_4 analogue with the aminobenzoyl group at the 2-position of Ins(1,3,4,5) P_4 inhibited the hydrolysis of 5-phosphate of [^3H]Ins(1,3,4,5) P_4 catalysed by erythrocyte ghosts, with a lower K_i value than seen with Ins(1,3,4,5) P_4 , whereas the analogue with the aminocyclohexanecarbonyl group at the same position had a higher K_i value. The Ins(1,4,5) P_3 analogues that we had previously synthesized were also capable of inhibiting this process, with the same tendency as Ins(1,3,4,5) P_4 analogues. Such differences in the potency among Ins(1,3,4,5) P_4 and Ins(1,4,5) P_3 analogues were applicable to other phosphatase activities, namely [^3H]Ins(1,3,4,5) P_4 3-phosphatase and [^3H]Ins(1,4,5) P_3 5-phosphatase. These results suggest that the active sites of these enzymes may catalyse the dephosphorylation in a similar fashion.

INTRODUCTION

Inositol 1,3,4,5-tetrakisphosphate [$\text{Ins}(1,3,4,5)\text{P}_4$], a product of the specific phosphorylation of inositol 1,4,5-trisphosphate [$\text{Ins}(1,4,5)\text{P}_3$], is a putative candidate for regulating Ca^{2+} homeostasis within the cell interior (Irvine *et al.*, 1988; Irvine, 1989). Irvine & Moore (1986, 1987) were the first to demonstrate that Ins(1,3,4,5) P_4 may be involved in the regulation of Ca^{2+} concentration, in conjunction with Ins(1,4,5) P_3 , inside sea-urchin eggs, possibly by stimulating the influx of Ca^{2+} through the plasma membrane. Petersen and colleagues reported that Ins(1,3,4,5) P_4 together with Ins(1,4,5) P_3 evoke in rat lacrimal acinar cells a sustained increase in the Ca^{2+} -activated K^+ current, depending on the external Ca^{2+} , and that this effect is assumed to be due to the linking of Ins(1,4,5) P_3 -sensitive and -insensitive Ca^{2+} pools (Morris *et al.*, 1987; Changya *et al.*, 1989). Higashida & Brown (1986) stated that Ins(1,3,4,5) P_4 by itself increased the non-specific cation conductance in the plasma membrane of neuroblastoma $\times$ glioma hybrid cells. In addition, there have been reports that Ins(1,3,4,5) P_4 alone evokes the mobilization of Ca^{2+} from internal Ca^{2+} stores in *Xenopus* eggs (Parker & Miledi, 1987; Snyder *et al.*, 1988) and in the rat cerebellum (Joseph *et al.*, 1989), albeit with a lesser potency. The latter authors excluded the possibility that Ins(1,3,4,5) P_4 acts through conversion into Ins(1,4,5) P_3 by activating Ins(1,3,4,5) P_4 3-phosphatase (Joseph *et al.*, 1989). Also, there is a report that Ins(1,3,4,5) P_4 alone induces sequestration of Ca^{2+} into Ca^{2+} stores of rat liver epithelial cells (261B) (Hill *et al.*, 1988). Thus far, a general consensus concerning the function of Ins(1,3,4,5) P_4 has not been reached. Nevertheless, Ins(1,3,4,5) P_4 is expected to exert effects on Ca^{2+} homeostasis through specific binding site(s) for Ins(1,3,4,5) P_4 in the microsomal fraction in a variety of cell types (Theibert *et al.*, 1987; Bradford & Irvine, 1987; Enyedi & Williams, 1988; Donie & Reiser, 1989).

Ins(1,3,4,5) P_4 is metabolized by two dephosphorylation routes: one is catalysed by Ins(1,3,4,5) P_4 5-phosphatase to produce Ins(1,3,4) P_3 , which is subsequently degraded to Ins(3,4) P_2 or Ins(1,3) P_2 by the respective phosphatase, and the other is by Ins(1,3,4,5) P_4 3-phosphatase to yield Ins(1,4,5) P_3 (Doughney *et al.*, 1988; Cunha-Melo *et al.*, 1988). Thus three potential sites

are listed as Ins(1,3,4,5) P_4 -recognizing ones: Ins(1,3,4,5) P_4 -binding sites that may possibly be involved in the function of Ins(1,3,4,5) P_4 as described above and two sites responsible for Ins(1,3,4,5) P_4 hydrolysis. Comprehension at the molecular level of the manner in which Ins(1,3,4,5) P_4 is recognized by interacting sites is crucial for elucidation of the function. It seems important to build up structure–activity profiles for the interaction of Ins(1,3,4,5) P_4 with binding sites and with metabolic enzymes, by using chemically modified molecules of Ins(1,3,4,5) P_4 , as was done with Ins(1,4,5) P_3 (Polokoff *et al.*, 1988; Hirata *et al.*, 1989, 1990).

We synthesized a series of Ins(1,4,5) P_3 analogues, in which the bulky substituent such as 4-azidobenzoyl (designated as analogue 195), 4-(5-benzamidoethyl-2-hydroxyphenylazo)benzoyl (204), 4-aminocyclohexanecarbonyl (206) or 4-aminobenzoyl group (209) was coupled with the 2-hydroxy group of Ins(1,4,5) P_3 . Using these analogues, we found that such modifications reduced little the ability of the analogues to interact with receptors and with metabolic enzymes (Hirata *et al.*, 1989, 1990). We have now synthesized two types of Ins(1,3,4,5) P_4 analogue that correspond to Ins(1,4,5) P_3 analogues 206 and 209, and their effects on [^3H]Ins(1,3,4,5) P_4 5-phosphatase and [^3H]Ins(1,3,4,5) P_4 3-phosphatase as well as [^3H]Ins(1,4,5) P_3 5-phosphatase were examined.

MATERIALS AND METHODS

Materials

[^3H]Ins(1,4,5) P_3 (specific radioactivity 754.8 GBq/mmol) and [^3H]Ins(1,3,4,5) P_4 (specific radioactivity 629 GBq/mmol) were obtained from DuPont–New England Nuclear. D-Ins(1,4,5) P_3 and DL-Ins(1,3,4,5) P_4 were chemically synthesized as described by Ozaki *et al.* (1986, 1987). D-Ins(1,3,4,5) P_4 was obtained from Calbiochem Corp. All other reagents were of the highest grade available.

Chemical synthesis of Ins(1,3,4,5) P_4 analogues

2-O-(4-Aminobenzoyl)-myo-inositol 1,3,4,5-tetrakisphosphate (KG-194). A solution of 6-O-benzyl-1,3,4,5-tetrakis-(1',5'-dihydrobenzodioxaphosphelynyl)-2-O-(4'-nitrobenzoyl)-myo-

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Fig. 1. Structures of Ins(1,3,4,5) P_4 , Ins(1,4,5) P_3 and their synthetic analogues

The compounds are racemic mixtures, except for Ins(1,4,5) P_3 .

inositol (271 mg) (S. Ozaki, Y. Watanabe & Y. Koga, unpublished work) and 5% palladium/carbon (Pd/C) (20 mg) in 10 ml of methanol/water (4:1, v/v) was stirred under H_2 gas for 24 h. After filtration of the mixture to remove Pd/C, the filtrate was evaporated and chromatographed on an Avicel column (CC-31) to afford KG-194 (79.9 mg, 50%).

2-O-(4-Aminocyclohexanecarbonyl)-myo-inositol 1,3,4,5-tetrakisphosphate (KG-210). A mixture of KG-194 (14.9 mg, 0.0221 mmol) and ruthenium oxide (21.5 mg, 0.162 mmol) in water (3.75 ml) and H_2 gas [8 MPa (80 atm)] were charged in a 50 ml autoclave and heated at 60 °C for 2 h. The catalyst was filtered and the filtrate was evaporated. The residue was chromatographed on a cellulose column with as eluent [propan-1-ol/conc. aq. NH_3 /water (5:4:1, by vol.)] to give KG-210 (14.3 mg, 96.8%; R_F 0.24).

Assays with human erythrocyte ghosts

Human erythrocyte ghosts were prepared from human blood (Downes *et al.*, 1982) and assayed for [3H]Ins(1,3,4,5) P_4 phosphatase or [3H]Ins(1,4,5) P_3 phosphatase activity. [3H]Ins(1,3,4,5) P_4 5-phosphatase activity was assayed as follows. The assay mixture (0.2 ml) contained 20 mM-Hepes buffer, pH 7.0, 5 mM-MgCl $_2$, 0.2 mg of saponin/ml and 1 μ M-D-Ins(1,3,4,5) P_4 {containing 370 Bq of [3H]Ins(1,3,4,5) P_4 }. After preincubation at 37 °C for 2 min, the reaction was initiated by adding approx. 150 μ g of erythrocyte ghosts; it was left to run for 15 min, then stopped by adding the same volume of 20% (w/v) trichloroacetic acid. After deproteinization and extraction with diethyl ether (3 $\times$ 3 ml), the production of [3H]Ins(1,3,4) P_3 and reduction of [3H]Ins(1,3,4,5) P_4 was analysed by applying the sample to a SAX column of an h.p.l.c. system. [3H]Ins(1,3,4,5) P_4 3-phosphatase activity was assayed by the same procedure as described above, except that 2 mM-EDTA in place of MgCl $_2$ was included, and the incubation was continued for up to 40 min. The enzyme activity was determined from the production of [3H]Ins(1,4,5) P_3 , which could easily be separated from [3H]Ins(1,3,4) P_3 , the other product of Ins(1,3,4,5) P_4 hydrolysis, by SAX column chromatography. There was no activation of [3H]Ins(1,3,4,5) P_4 5-phosphatase activity to produce [3H]Ins(1,3,4) P_3 in the absence of Mg $^{2+}$. [3H]Ins(1,4,5) P_3 5-phosphatase activity was assayed by the same procedure as described for [3H]Ins(1,3,4,5) P_4 5-phosphatase, except that 1 μ M-D-Ins(1,3,4,5) P_4 and [3H]Ins(1,3,4,5) P_4 were replaced with 10 μ M-D-Ins(1,4,5) P_3 and [3H]Ins(1,4,5) P_3 with the same radioactivity respectively. Erythrocyte ghosts were also

Fig. 2. Inhibition of [3H]Ins(1,3,4,5) P_4 5-phosphatase, [3H]Ins(1,3,4,5) P_4 3-phosphatase and Ins(1,4,5) P_3 5-phosphatase activities by analogues of Ins(1,3,4,5) P_4 and Ins(1,4,5) P_3

Erythrocyte ghosts (approx. 150 μ g) were incubated with either 1 μ M-[3H]Ins(1,3,4,5) P_4 or 10 μ M-[3H]Ins(1,4,5) P_3 at 37 °C in the presence of unlabelled Ins(1,3,4,5) P_4 or Ins(1,4,5) P_3 analogues at concentrations indicated on the abscissa. The results are expressed relative to that in the absence of unlabelled analogues. ●, Ins(1,3,4,5) P_4 ; ▲, KG-194; ■, KG-210; ○, Ins(1,4,5) P_3 ; △, 209; □, 206. (a) [3H]Ins(1,3,4,5) P_4 5-phosphatase activity was assayed in the presence of Mg $^{2+}$ for 15 min. Each point is the mean of three determinations done in duplicate. (b) [3H]Ins(1,3,4,5) P_4 3-phosphatase activity was assayed in the absence of Mg $^{2+}$ for 40 min. Each point is the mean of two determinations. (c) [3H]Ins(1,4,5) P_3 5-phosphatase activity was assayed in the presence of Mg $^{2+}$ for 15 min. Each point is the mean of three determinations done in duplicate.

used as the phosphatase to examine whether Ins(1,3,4,5) P_4 analogues can serve as substrate: the ghosts (approx. 0.34 mg) were incubated with Ins(1,3,4,5) P_4 analogues at a concentration of 10 μ M in the solution (300 μ l) containing 20 mM-Hepes buffer, pH 7.0, 5 mM-MgCl $_2$, 0.2 mg of saponin/ml and 20 mM-LiCl at 37 °C for 60 min. Li $^+$ was included to block the 1-phosphatase activity towards Ins(1,3,4) P_3 , a product of Ins(1,3,4,5) P_4 5-hydrolysis, but actually there was no difference in the results with or without Li $^+$. The reaction was terminated by adding 300 μ l of

Table 1. Potency of Ins(1,3,4,5) P_4 analogues in Ins(1,3,4,5) P_4 5-phosphatase and Ins(1,4,5) P_3 5-phosphatase activities

The apparent K_i values were determined from Dixon plots. Each value is the mean of two experiments. The IC_{50} values are from Fig. 2(b). For the hydrolysis by Ins(1,3,4,5) P_4 5-phosphatase, erythrocyte ghosts (0.34 mg) were incubated with 10 μ M-Ins(1,3,4,5) P_4 analogues at 37 °C for 60 min. Each value is the mean of three experiments done in duplicate.

Compound	K_i with [3 H]Ins(1,3,4,5) P_4 5-phosphatase (μ M)	IC_{50} with [3 H]Ins(1,3,4,5) P_4 3-phosphatase (μ M)	K_i with [3 H]Ins(1,4,5) P_3 5-phosphatase (μ M)	Hydrolysis by Ins(1,3,4,5) P_4 5-phosphatase (nmol)
Ins(1,3,4,5) P_4	5.2	2.8	2.5	1.1
KG-194	0.8	1.4	0.7	0.70
KG-210	12.8	3.3	6	0.74

20 % trichloroacetic acid. After deproteinization, the supernatant was assayed for inorganic phosphate (Youngburg & Youngburg, 1930).

RESULTS

Two types of Ins(1,3,4,5) P_4 analogues were synthesized. The structures of the compounds, including Ins(1,4,5) P_3 analogues (Hirata *et al.*, 1989, 1990), are shown in Fig. 1.

Human erythrocyte ghosts were used as the enzyme source for [3 H]Ins(1,3,4,5) P_4 phosphatases and [3 H]Ins(1,4,5) P_3 phosphatases. First, the [3 H]Ins(1,3,4,5) P_4 5-phosphatase activity was found to have a K_m value of 2.5 μ M and a V_{max} of 0.27 nmol/mg per min (mean of four determinations), as assessed by a Lineweaver–Burk plot analysis (results not shown). The hydrolysis of [3 H]Ins(1,3,4,5) P_4 at 1 μ M was dose-dependently inhibited by the Ins(1,3,4,5) P_4 analogues, as well as by Ins(1,3,4,5) P_4 (Fig. 2a). A Dixon plot analysis of the inhibition revealed that the Ins(1,3,4,5) P_4 analogues behaved like apparent competitive inhibitors (Table 1); thus these analogues probably compete with [3 H]Ins(1,3,4,5) P_4 for the active site of the phosphatase. An analogue KG-194, with an aminobenzoyl group on the second carbon atom of Ins(1,3,4,5) P_4 , was more potent than Ins(1,3,4,5) P_4 , whereas KG-210 with an aminocyclohexanecarbonyl group was less potent. Such a difference in potency was the same as seen with Ins(1,4,5) P_3 analogues in [3 H]Ins(1,4,5) P_3 5-phosphatase assay (Hirata *et al.*, 1989, 1990; see also Fig. 2c). Each analogue at 10 μ M was incubated with erythrocyte ghosts at 37 °C for 60 min, to observe whether they could serve as substrate. Both analogues could be hydrolysed, though the extent of the hydrolysis was less than that seen with Ins(1,3,4,5) P_4 (Table 1).

Ins(1,4,5) P_3 and its analogues 209 and 206, with the aminobenzoyl group and aminocyclohexanecarbonyl group respectively, were also effective in inhibiting [3 H]Ins(1,3,4,5) P_4 5-phosphatase activity (Fig. 2a). The order of potency among Ins(1,4,5) P_3 and its analogues regarding the inhibition was the same as that among Ins(1,3,4,5) P_4 analogues, i.e. that of the analogue with the aminobenzoyl group was greater than that of Ins(1,4,5) P_3 or Ins(1,3,4,5) P_4 , whereas that of the analogue with the aminocyclohexanecarbonyl group was less.

Human erythrocyte ghosts were then assayed in the absence of Mg^{2+} for [3 H]Ins(1,3,4,5) P_4 3-phosphatase activity. The kinetic parameters, as assessed by Lineweaver–Burk plots of the [3 H]Ins(1,3,4,5) P_4 3-phosphatase, were 0.6 μ M or 7.4 pmol/mg per min for K_m or V_{max} , respectively, which were 1/4.2 or 1/36 respectively of the values for the [3 H]Ins(1,3,4,5) P_4 5-phosphatase activity. Fig. 2(b) shows the dose-dependence of Ins(1,3,4,5) P_4 analogues and Ins(1,4,5) P_3 analogues in inhibiting the hydrolysis of [3 H]Ins(1,3,4,5) P_4 catalysed by [3 H]Ins(1,3,4,5) P_4 3-phosphatase or erythrocyte ghosts. All of the analogues were capable of

inhibiting the hydrolysis. The order of the potency by a series of Ins(1,3,4,5) P_4 analogues and Ins(1,4,5) P_3 analogues was much the same as seen for Ins(1,3,4,5) P_4 5-phosphatase activity (see Table 1).

The Ins(1,3,4,5) P_4 analogues were also examined for possible effects regarding inhibition of [3 H]Ins(1,4,5) P_3 5-phosphatase activity. Indeed, they were capable of inhibiting the hydrolysis, in a competitive manner (Fig. 2c and Table 1). The analogues with the aminobenzoyl group in both Ins(1,3,4,5) P_4 and Ins(1,4,5) P_3 are most potent, as was noted for other phosphatase activities.

DISCUSSION

Ins(1,3,4,5) P_4 can be hydrolysed by at least two routes: the activation of Ins(1,3,4,5) P_4 5-phosphatase or Ins(1,3,4,5) P_4 3-phosphatase yields Ins(1,3,4) P_3 or Ins(1,4,5) P_3 respectively. Ins(1,3,4,5) P_4 5-phosphatase activity was first demonstrated with erythrocyte membranes to show that Ins(1,3,4,5) P_4 is the precursor of Ins(1,3,4) P_3 (Batty *et al.*, 1985). Later, Connolly *et al.* (1987) showed that Ins(1,4,5) P_3 5-phosphatase purified from human platelets also attacks Ins(1,3,4,5) P_4 , thereby indicating that a single enzyme catalysed the dephosphorylation of both Ins(1,3,4,5) P_4 and Ins(1,4,5) P_3 . Such was confirmed by the reports by Hansen *et al.* (1987), with rat brain, by Takimoto *et al.* (1989), with the same tissue, and by Erneux *et al.* (1989), with bovine brain. Furthermore Ins(1,3,4,5) P_4 competed with Ins(1,4,5) P_3 for the active site of purified Ins(1,4,5) P_3 5-phosphatase from human platelets, with a K_i value of 0.5 μ M, a value similar to the K_m for the hydrolysis of Ins(1,3,4,5) P_4 by the same enzyme (Connolly *et al.*, 1987). Thus the single active site of the single enzyme may recognize both substrates, Ins(1,3,4,5) P_4 and Ins(1,4,5) P_3 . The results obtained here favour the above view: not only Ins(1,3,4,5) P_4 analogues but also Ins(1,4,5) P_3 analogues competed for [3 H]Ins(1,3,4,5) P_4 5-phosphatase, with a potency similar to that seen for [3 H]Ins(1,4,5) P_3 5-phosphatase. Addition of the aminobenzoyl group at the 2-position of Ins(1,3,4,5) P_4 or Ins(1,4,5) P_3 increased the affinity for the active site of the phosphatase, but substitution of the benzene ring with that of cyclohexane attenuated the increased affinity for the active site of the enzyme. Thus even a simple difference in structure can cause a large difference in the potency and this may provide a clue as to the active site of the enzyme.

Ins(1,3,4,5) P_4 3-phosphatase was first demonstrated in erythrocyte ghosts by Doughney *et al.* (1988) and independently in rat basophilic leukaemia (RBL-2H3) cells by Cunha-Melo *et al.* (1988). They did not draw any conclusion as to whether the 3-phosphatase activity is the same enzyme as the 5-phosphatase with altered specificity in the absence of Mg^{2+} , or a distinct enzyme, the activity of which is masked by the 5-phosphatase at millimolar Mg^{2+} , but is more active in the absence of Mg^{2+} . More recent reports support the latter view that two phosphatase

activities are catalysed by distinct enzymes: Hughes & Shears (1990) reported the different sensitivities to inositol pentakis- and hexakis-phosphates of 3-phosphatase and 5-phosphatase activities in the rat parotid glands, and Höer *et al.* (1990) reported that Mg^{2+} increases 5-phosphatase activity without proportionally decreasing that of the 3-phosphatase in pig brain. Furthermore Hodgson & Shears (1990) reported the chromatographic separation of the rat liver enzymes on an anion-exchange column. However, [3H]Ins(1,3,4,5) P_4 3-phosphatase activity was also inhibited by both Ins(1,3,4,5) P_4 analogues and Ins(1,4,5) P_3 analogues, with similar potencies to those for [3H]Ins(1,3,4,5) P_4 5-phosphatase and [3H]Ins(1,4,5) P_3 5-phosphatase. Furthermore the analogue with the aminobenzoyl group at 2-position was more potent than that with the aminocyclohexanecarbonyl group. Thus these results suggest that the active sites of these two distinct enzymes for Ins(1,3,4,5) P_4 may be similar.

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