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Differential stereoselectivity of D- and L-*myo*-inositol 1,2,4,5-tetrakisphosphate binding to the inositol 1,4,5-trisphosphate receptor and 3-kinase

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Abstract

D- and L-*myo*-inositol 1,2,4,5-tetrakisphosphate ($Ins(1,2,4,5)P_4$) were investigated for their ability to bind to the D-*myo*-inositol 1,4,5-trisphosphate ($Ins(1,4,5)P_3$) receptor in a bovine adrenal cortical membrane fraction, to mobilize intracellular Ca^{2+} stores in *Xenopus* oocytes, and to bind to the rat brain $Ins(1,4,5)P_3$ 3-kinase overexpressed and purified in *E. coli*. In competitive binding experiments with the $Ins(1,4,5)P_3$ receptor, D-$Ins(1,2,4,5)P_4$ effectively displaced [^{3}H]$Ins(1,4,5)P_3$ in a concentration-dependent manner with a potency comparable to that of D-$Ins(1,4,5)P_3$, while L-$Ins(1,2,4,5)P_4$ was ~50-fold less effective than D-$Ins(1,4,5)P_3$ and D-$Ins(1,2,4,5)P_4$. The DL-$Ins(1,2,4,5)P_4$ racemate bound to the $Ins(1,4,5)P_3$ receptor with an apparent intermediate efficiency. Injection of D-$Ins(1,2,4,5)P_4$ into oocytes evoked a chloride current dependent on intracellular Ca^{2+} mobilization in which the agonists ranked in a similar order of potency as in the $Ins(1,4,5)P_3$ receptor binding. On the other hand, D-$Ins(1,2,4,5)P_4$ only inhibited the binding of [^{3}H]$Ins(1,4,5)P_3$ to 3-kinase very weakly with a markedly reduced potency compared to D-$Ins(1,4,5)P_3$, indicating that D-$Ins(1,2,4,5)P_4$ is not an effective competitor in the phosphorylation of [^{3}H]-$Ins(1,4,5)P_3$ by 3-kinase. The results, therefore, clearly indicate that D-$Ins(1,2,4,5)P_4$ is as effective as D-$Ins(1,4,5)P_3$ in the binding to the receptor but not 3-kinase, and access of $Ins(1,2,4,5)P_4$ over the $Ins(1,4,5)P_3$ receptor calls for stringent stereospecificity with D-$Ins(1,2,4,5)P_4$ being the active form in DL-$Ins(1,2,4,5)P_4$-mediated Ca^{2+} mobilization.

Keywords: D-*myo*-inositol 1,2,4,5-tetrakisphosphate (1,2,4,5)P_4); $Ins(1,4,5)P_3$ receptor; $Ins(1,4,5)P_3$ 3-kinase; Calcium mobilization

1. Introduction

Stimulation of cell-surface phospholipase C-coupled receptors initiates hydrolysis of the membrane-bound lipid phosphatidylinositol 4,5-bisphosphate by action of phospholipase C. In the process at least two second messengers, diacylglycerol and *myo*-inositol 1,4,5-trisphosphate ($Ins(1,4,5)P_3$), are produced. Diacylglycerol stimulates protein kinase C, whereas $Ins(1,4,5)P_3$ releases calcium from non-mitochondrial intracellular stores via interactions with $Ins(1,4,5)P_3$ receptors (Berridge, 1998). $Ins(1,4,5)P_3$ is then rapidly metabolized by either $Ins(1,4,5)P_3$ 5-phosphatase which catalyzes dephosphorylation of $Ins(1,4,5)P_3$ to yield $Ins(1,4)P_2$, or by $Ins(1,4,5)P_3$ 3-kinase which catalyzes phosphorylation of the 3-hydroxyl group to generate *myo*-inositol 1,3,4,5-tetrakisphosphate ($Ins(1,3,4,5)P_4$) (Communi et al., 1995).

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Although controversy exists about the role of Ins(1,3,4,5)P_4 during cell signaling, Ins(1,3,4,5)P_4 could act as a second messenger mediating the entry of extracellular Ca^{2+} through plasma membrane ion channels and the mobilization of Ca^{2+} from the intracellular calcium stores, albeit less potently than Ins(1,4,5)P_3 (Wilcox et al., 1993b).

Previous studies have shown that the Ins(1,4,5)P_3 receptor and the 3-kinase both exhibit a remarkable stereo- and regio-selectivity. For example, L-Ins(1,4,5)P_3 and L-Ins(1,3,4,5)P_4 have an approximately 100–1000-fold lower affinity for Ins(1,4,5)P_3 binding sites in pig cerebellum in comparison to D-Ins(1,4,5)P_3 and D-Ins(1,3,4,5)P_4, respectively (Wilcox et al., 1993a). L-Ins(1,4,5)P_3 was known to be 50–100-fold less potent than D-Ins(1,4,5)P_3 in binding to the 3-kinase (Polokoff et al., 1988). In addition, D-Ins(1,3,6)P_3, which has similar dispositions of the three phosphate groups but inverted stereochemistry at the 2- and 4-OH, did not inhibit the 3-kinase activity (Choi et al., 1997). This means that there is a structural component in the recognition of inositol phosphate regioisomers by the binding proteins. More detailed information about the binding activity is therefore important, in particular, when trying to develop either inhibitors or activators of the binding proteins.

Recent studies of all possible regioisomers of the inositol phosphates in terms of their ability to bind to the Ins(1,4,5)P_3 receptor and to mobilize Ca^{2+} from Ins(1,4,5)P_3-sensitive Ca^{2+} stores revealed that a synthetic compound Ins(1,2,4,5)P_4 was effective in receptor binding and Ca^{2+} mobilization albeit 2–3 times less potently than the biological ligand D-Ins(1,4,5)P_3 (Burford et al., 1997; Mills et al., 1993; Wilcox et al., 1994). At present, synthesis of Ins(1,2,4,5)P_4 in racemic form (Carless and Busia, 1990; Meek et al., 1988), chiral D- (Kozikowski et al., 1995), and L-form (Mills and Potter, 1997) has been reported. Many studies using the racemate Ins(1,2,4,5)P_4 indicated that DL-Ins(1,2,4,5)P_4 is an active analogue of Ins(1,4,5)P_3, but little is known about differences between chiral D- and L-forms as of yet. We, therefore, assessed the effects of the possible stereoisomers of synthetic Ins(1,2,4,5)P_4 on biological activities such as the binding to the Ins(1,4,5)P_3 receptors, the intracellular Ca^{2+} mobilization, and the binding to the Ins(1,4,5)P_3 3-kinase. Our results present evidence that D-Ins(1,2,4,5)P_4 has differential binding effects on the interaction with Ins(1,4,5)P_3 receptor and Ins(1,4,5)P_3 3-kinase and the binding of Ins(1,2,4,5)P_4 to Ins(1,4,5)P_3-specific binding sites exhibits stereospecificity and the activity of the racemate DL-Ins(1,2,4,5)P_4 comes virtually all from the D-Ins(1,2,4,5)P_4 isomer rather than L-Ins(1,2,4,5)P_4 isomer.

2. Experimental procedures

2.1. Materials

Trizma base, EGTA, trichloroacetic acid (TCA), HEPES, Ins(1,4,5)P_3, leupeptin, calpain inhibitors I and II, aprotinin, and phenylmethylsulfonyl fluoride were obtained from Sigma Chemical Co. (St Louis, MO, USA). Dithiothreitol was purchased from Research Biochemicals Inc. (Natick, MA, USA) and [^{3}H]Ins(1,4,5)P_3 from DuPont NEN Research Products (Boston, MA, USA). The detailed synthetic routes for the inositol phosphates regioisomers used in the experiments were reported previously (Chung and Chang, 1995, Chung et al., 1998) and the compounds were diluted in ultrapure water.

2.2. Preparation of the bovine adrenal cortical membrane fraction

The Ins(1,4,5)P_3 binding protein was prepared from bovine adrenal cortex according to the method of Suh et al. (1995). Briefly, the adrenal glands were demedullated and decapsulated, and the remaining cortex was homogenized in a buffer (20 mM $NaHCO_3$ and 1 mM dithiothreitol, pH 8.0). The homogenate was centrifuged at 5000 × g for 15 min at 4°C. The pellet was resuspended and centrifuged at 38,000 × g for 20 min at 4°C. The pellet was finally resuspended at a concentration of about 4 mg of protein/ml, determined by the Bradford method, and the sample was stored at −70°C until use.

2.3. Binding assay of Ins(1,4,5)P_3 and Ins(1,2,4,5)P_4

Increasing concentrations (1–12,000 nM) of Ins(1,4,5)P_3 and other polyphosphates were incubated with 1.25 nM [^{3}H]Ins(1,4,5)P_3 in a buffer containing 25 mM Tris/HCl, pH 8.0, 5 mM $NaHCO_3$, 1 mM EDTA and 250 mM dithiothreitol in a total assay volume of 80 μl. Incubations were initiated by the addition of about 80 μg of adrenal cortical membrane protein and continued for 15 min at 4°C to achieve equilibrium binding as previous described (Challiss et al., 1990). Bound and free ligands were separated by centrifugation (12,000 × g, 5 min). The pellets were then dissolved in 2% SDS and the radioactivity of [^{3}H]Ins(1,4,5)P_3 was measured using the liquid scintillation analyzer (Packard, Canberra Com., Groningen, Netherlands). Residual bound radioactivity in the presence of 10 μM D-Ins(1,4,5)P_3 was defined as non-specific binding.

2.4. Oocyte preparation and microinjection

Individual *Xenopus* oocyte at stage VI (Dumont,

1972), developmentally full-grown stage reached about 1.2 mm in diameter, were manually dissected from their outer follicles using watchmaker's forceps and stored in Modified Barth Saline (MBS: 88 mM NaCl, 1 mM KCl, 2.4 mM $NaHCO_3$, 0.33 mM $Ca(NO_3)_2$, 0.41 mM $CaCl_2$, 10 mM Na–HEPES, pH 7.4) at 19°C and used within 1 day after isolation. All inositol phosphates were dissolved in deionized distilled water as a concentration of 1 mM, and the indicated volumes were microinjected into the oocyte using a Nanoliter Injector (WPI, Sarasota, FL, USA).

2.5. Voltage-clamp recording

Currents elicited by the injection of inositol phosphates were recorded at −70 mV holding potential of oocyte membrane using the standard double electrode voltage-clamp technique. A single oocyte was placed into the recording chamber filled with 1.5 ml MBS. Microelectrodes were pulled in one stage from capillary glass (Borosilicate Glass Capillaries with an inner filament, Warner corporation, New Haven, CT, USA) on a micropipette puller (model 700C; David Kopf Instruments, Tujunga, CA, USA). They were filled with 3 M KCl, and tip resistances were usually 1–5 MΩ. The cell was voltage clamped using a two-microelectrode voltage clamp amplifier (oocyte clamp OC725A; Warner Corp. New Haven, CT, USA), connected to a data acquisition system, MacLab/4e (AD Instruments Pty Ltd., Castle Hill, Australia) running on a Power Macintosh computer. Membrane currents were sampled at 4 Hz. To minimize variations among oocytes from different females, the comparisons of the current responses were made in oocytes from the same animal. All experiments were performed at room temperature.

2.6. Assay of Ins(1,4,5)P_3 3-kinase activity

The rat brain Ins(1,4,5)P_3 3-kinase was overexpressed in *E. coli* and purified to near homogeneity. This 3-kinase showed similar kinetic and immunological properties to the enzyme purified from rat brain (data not shown) and used as the enzyme source for the activity assay. The activity of 3-kinase was assayed as described previously (Lee et al., 1990) with a slight modification. The reaction was initiated by addition of 10 μl of purified 3-kinase to the 190 μl of reaction mixture consisting of 50 mM Tris–Cl, pH 8.0, 100 mM KCl, 2 mM EGTA, 5 mM ATP, 1 mM DTT, 1 mM 2,3-DPG, 1 μM D-Ins(1,4,5)P_3, 20,000 dpm of [^{3}H]-Ins(1,4,5)P_3, and increasing amount of synthetic inositol phosphates. The reaction mixture was gently vortexed and incubated at 37°C for 10 min. The reaction was terminated by adding 50 ml of 1 M H_3PO_4. The terminated reaction mixture was neutralized by the addition of 50 ml of 1 M KOH and diluted with 500 ml of water. Each sample was immediately applied to an anion exchange column containing 500 ml of Dowex AG1-X8 (formate form, 100–200 mesh) and then subsequently washed 10 times with 2 ml of 0.55 M ammonium formate in 0.1 M formic acid to remove the unreacted D-Ins(1,4,5)P_3. The product of 3-kinase, [^{3}H]-Ins(1,3,4,5)P_4, was then eluted with 1.5 ml of 1.5 M ammonium formate in 0.1 M formic acid and then mixed with 10 ml of a complete counting cocktail solution (Budget-Solve, Research Product International Corp.) and its radioactivity was counted in a liquid scintillation counter.

2.7. Analysis of data

Inhibition constant (IC_{50}) values for the Ins(1,4,5)P_3 binding curves were converted to K_i values using the Cheng and Prusoff (1973) equation. All data are expressed as the means (±standard error) of 3–5 independent experiments.

3. Results and discussion

D-Ins(1,4,5)P_3 receptors prepared from bovine adrenal cortex were assayed for D-[^{3}H]Ins(1,4,5)P_3 binding to determine whether or not the receptor distinguishes between the stereospecificity of D- and L-Ins(1,2,4,5)P_4.

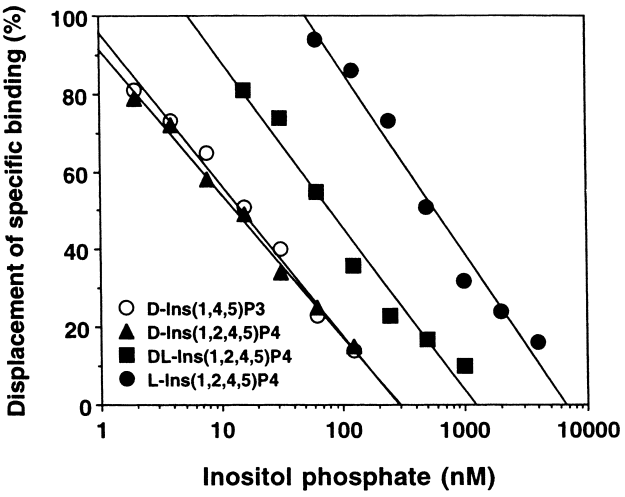

Fig. 1. Inhibition of specific [^{3}H]Ins(1,4,5)P_3 binding to the bovine adrenal cortical membrane fraction at increasing concentrations (1–12,000 nM) of inositol polyphosphates. The membrane fraction was incubated with 1.25 nM [^{3}H]Ins(1,4,5)P_3 in the presence of various concentrations of D-Ins(1,4,5)P_3, D-Ins(1,2,4,5)P_4, L-Ins(1,2,4,5)P_4, and DL-Ins(1,2,4,5)P_4. Residual bound radioactivity in the presence of 10 μM D-Ins(1,4,5)P_3 was defined as nonspecific binding (about 5% of total binding) and was subtracted from the total binding. The specific binding in the absence of unlabeled ligand was taken as 100%. Each point represents the mean of four determinants.

Fig. 1 shows that D-Ins(1,2,4,5)P_4 inhibited [^{3}H]Ins(1,4,5)P_3 binding in a concentration-dependent manner with a K_i of 13.5 which was almost the same value as the one obtained with D-Ins(1,4,5)P_3 (K_i = 15.6) (see Table 1). Compared to D-Ins(1,2,4,5)P_4, L-Ins(1,2,4,5)P_4 exhibited an approximately 50-fold lower affinity (K_i = 599.4) for the Ins(1,4,5)P_3 receptor, indicating that the binding of Ins(1,2,4,5)P_4 to the Ins(1,4,5)P_3 receptor is highly stereospecific. On the other hand, the racemic DL-Ins(1,2,4,5)P_4 had an intermediate affinity (K_i = 82.6) for the receptor. Many other InsP_4 analogues showed more than 100-fold weaker displacement activity in comparison to D-Ins(1,4,5)P_3 and also an affinity weaker than that of L-Ins(1,2,4,5)P_4 (Table 1).

The mobilization of intracellular Ca^{2+} was assayed by measuring the amplitude of rapid chloride currents evoked in voltage-clamped *Xenopus* oocytes maintained in Ca^{2+}-free medium and microinjected with different inositol phosphates which amounted to 6.25–50 pmole/oocyte in final concentrations. The chloride currents are indirect indicators of intracellular Ca^{2+} changes, because these chloride channels are known to be Ca^{2+}-dependent (Ferguson et al., 1991). Fig. 2(A) shows that the most potent compound among the InsP_4 analogues was D-Ins(1,2,4,5)P_4 which raised the chloride current as potently as Ins(1,4,5)P_3. However, DL-Ins(1,2,4,5)P_4 failed to achieve the same level of chloride current. L-Ins(1,2,4,5)P_4 did not evoke a significant chloride current even at concentrations of up to 50 pmole/oocyte. These results indicate that the effects of DL-Ins(1,2,4,5)P_4 on binding to the Ins(1,4,5)P_3 receptor and Ca^{2+} mobilization from intracellular stores observed in the previous studies (Burford et al., 1997; Mills et al., 1993; Wilcox et al., 1994) are mediated exclusively by the D-isomer. The chloride currents were induced by the isomers in a concentration-dependent manner up to the injection of 50 pmole per oocyte (Fig. 2(B)). DL-Ins(1,2,4,5)P_4 had about 50–60% of the potency of D-Ins(1,4,5)P_3 and D-Ins(1,2,4,5)P_4 in generating chloride current. The data may explain why other researchers using the racemic mixture have only observed a 50–70% response in Ca^{2+} release and binding affinity in comparison to D-Ins(1,4,5)P_3 (Burford et al., 1997; Wilcox et al., 1994). The effects of the various inositol phosphates on the Ca^{2+}-release-dependent chloride current and the binding to Ins(1,4,5)P_3 receptors are summarized in Table 1. D-Ins(1,2,4,5)P_4 was the most potent among the InsP_4 analogues tested with a potency similar to that of D-Ins(1,4,5)P_3. Other InsP_4 analogues had little or no effect on the Ca^{2+} mobilization, a result that parallels the receptor binding properties.

Table 1
Comparison of binding affinities and chloride currents triggered by various InsP_4 isomers[a]

Ins(1,4,5)P_3 analogues	K_i (nM)	Chloride current (nA)
D-Ins(1,4,5)P_3	15.6	1317 ± 108
D-Ins(1,2,4,5)P_4	13.5	1363 ± 117
DL-Ins(1,2,4,5)P_4	82.6	821 ± 69
L-Ins(1,2,4,5)P_4	599.4	69 ± 18
DL-Ins(1,2,4,6)P_4	908.5	57 ± 29
Ins(1,2,3,5)P_4	> 1000	(−)
Ins(1,3,4,6)P_4	> 1000	(−)
DL-Ins(1,4,5,6)P_4	> 4000	(−)
DL-Ins(1,2,5,6)P_4	> 1000	(−)
DL-Ins(1,2,3,4)P_4	> 1000	(−)

[a] K_i values were determined from the equation $K_i = IC_{50}/1 + ([radioligand]/K_D)$, where the equilibrium dissociation constant K_D of bovine adrenal cortical membranes (5.62×10^{-9} M) was used (Burford et al., 1997). Chloride currents in response to the injection of 50 pmoles of inositol phosphates into an each oocyte were measured as described in Section 2. The data are the means (±standard error) of three or four independent experiments. (−) means no current was detected after the injection of the indicated isomer (50 pmoles each) into an oocyte.

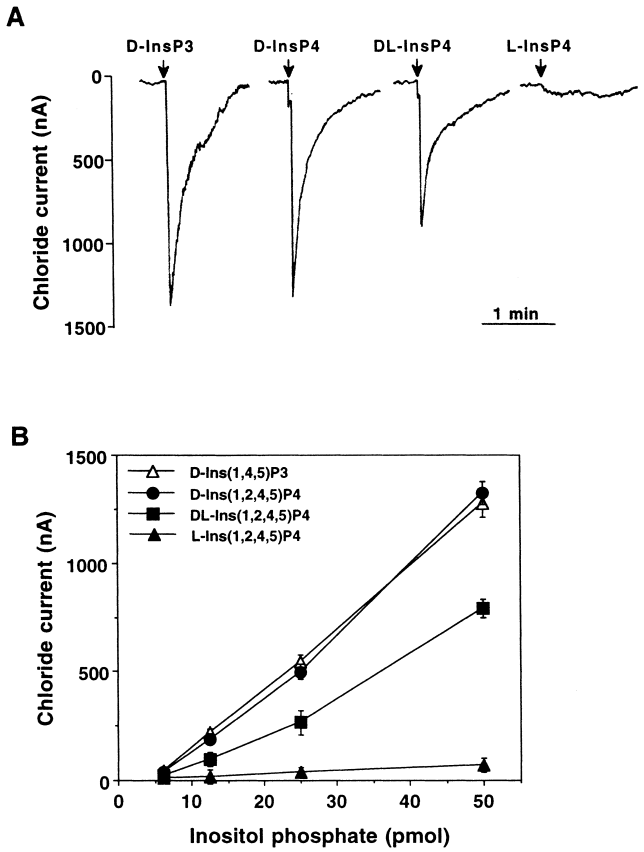

Fig. 2. Chloride currents triggered by the injection of inositol polyphosphates into stage VI oocytes. (A) Currents in response to the injection of 50 pmoles of D-Ins(1,4,5)P_3, D-Ins(1,2,4,5)P_4, DL-Ins(1,2,4,5)P_4, and L-Ins(1,2,4,5)P_4 were measured as described in Section 2. (B) Concentration-dependent increases in chloride currents were obtained by the injection of 6.25, 12.5, 25, and 50 pmoles of each inositol phosphate.

The effect of the D- and L-Ins(1,2,4,5)P_4 on the activity of Ins(1,4,5)P_3 3-kinase was examined to determine their binding to the 3-kinase. Addition of D-Ins(1,4,5)P_3 concentration-dependently inhibited the phosphorylation of D-[^{3}H]Ins(1,4,5)P_3 by 3-kinase with a mean IC_{50} value of 7.2 ± 0.5 μM (Fig. 3). However, D-Ins(1,2,4,5)P_4 weakly inhibited the phosphorylation of Ins(1,4,5)P_3 (< 25%) at 100 μM, the highest concentration examined, but the effect was not prominent compared to D-Ins(1,4,5)P_3, indicating that introduction of a bulky phosphate group at the 2-position of D-Ins(1,4,5)P_3 hinders binding to 3-kinase. On the other hand, L-Ins(1,2,4,5)P_4 did not show any significant inhibition on the 3-kinase activity. These results suggest that the recognition of *myo*-inositol phosphate regioisomers by 3-kinase is also structure-selective, and also suggest that there may be structural differences in a key binding site of 3-kinase compared to Ins(1,4,5)P_3 receptor. The crucial amino acid residues that form the core of the putative Ins(1,4,5)P_3 binding pocket on the Ins(1,4,5)P_3 receptor have been identified (Yoshikawa et al., 1996). Considering the above recent findings, pharmacological approaches using synthetic analogues of Ins(1,4,5)P_3 may help to map further the ligand binding site of the Ins(1,4,5)P_3 receptor and 3-kinase and elucidate the vital structure-activity information (Potter and Lampe, 1995). This information would be very useful for the rational design and development of novel synthetic ligands for the Ins(1,4,5)P_3 receptor and 3-kinase.

Many previous studies of structure-activity relationships have shown that there are minimal prerequisites that have to be met in the interactions of inositol phosphates with binding proteins. For example, early studies with permeabilized cells have indicated that the vicinal phosphates on position 4 and 5 of Ins(1,4,5)P_3 play a crucial role in Ca^{2+} release and that the monoester phosphate at position 1 contributes to the Ins(1,4,5)P_3 receptor binding specificity (Berridge and Irvine, 1984). It has been proposed that the hydroxyl groups at positions 2, 3, and 6 of Ins(1,4,5)P_3 might have the potential to form hydrogen bonds with the functional groups of receptor proteins and with neighboring phosphates (Potter and Lampe, 1995). Additionally, the present results suggest that the Ins(1,4,5)P_3 receptor seems to have a vacant space in the C-2 axial direction of D-Ins(1,4,5)P_3, since the presence of an additional phosphate to D-Ins(1,2,4,5)P_4 at C-2 position does not interfere with binding to the Ins(1,4,5)P_3 receptor or the Ca^{2+} release that results from this binding. On the other hand, it has been known that the configurations of hydroxyl group at 2, 3, and 6 position of D-Ins(1,4,5)P_3 was not critical to the binding to Ins(1,4,5)P_3 3-kinase, while all three phosphate groups of D-Ins(1,4,5)P_3 were essential for the efficient binding to 3-kinase since the activity of 3-kinase was very weakly inhibited by inositol monophosphates and inositol bisphosphates (Choi et al., 1997; Hirata et al., 1993). It was also reported that bulky neutral substituents such as benzoyl or methylbenzoyl groups at the C-2 position were well tolerated by 3-kinase and that 3-kinase may have a vacant space at the C-2 axial direction of D-Ins(1,4,5)P_3 (Hirata et al., 1995). However, our present data indicate that introduction of a bulky phosphate group at the 2-position in such as D-Ins(1,2,4,5)P_4 hinders the binding to 3-kinase and weakly inhibits the 3-kinase activity. Based on this observation the binding site of 3-kinase appears to have negative charges near the vacant space surrounding the C-2 position of D-Ins(1,4,5)P_3 and the negative-charged pocket might cause unfavorable interactions between 3-kinase and the 2-phosphate group of D-Ins(1,2,4,5)P_4. Recently, it has been shown that the steric factor of the substituent at the C-6 position of D-Ins(1,4,5)P_3 was also critical for binding to 3-kinase and either the stereoinversion or a bulky substituent at C-6 position was not tolerated (Choi et al., 1997).

In conclusion, we found that D-Ins(1,2,4,5)P_4 exhibits as much potency as Ins(1,4,5)P_3 in terms of Ins(1,4,5)P_3 receptor binding affinity and Ca^{2+} mobilization, while the L-isomer had little effect and the racemic mixture had an intermediate effect. On the other hand, D-Ins(1,2,4,5)P_4 did not bind to 3-kinase effectively compared to D-Ins(1,4,5)P_3. Since Ins(1,2,4,5)P_4 was differentially recognized by the 3-kinase and receptor our results also suggest that Ins(1,2,4,5)P_4 could be a lead compound in the

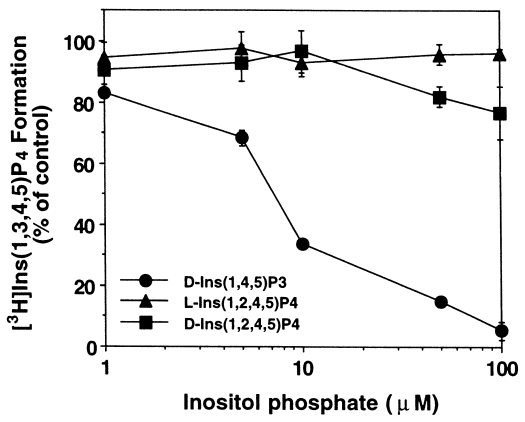

Fig. 3. Inhibition of Ins(1,4,5)P_3 3-kinase-mediated [^{3}H]Ins(1,3,4,5)P_4 production by synthetic inositol phosphates. Assays were performed with 2 μM D-[^{3}H]Ins(1,4,5)P_3 by increasing the concentration of D-Ins(1,4,5)P_3, D-Ins(1,2,4,5)P_4, or L-Ins(1,2,4,5)P_4 in the absence of Ca^{2+}. The *y*-axis represents the amount of [^{3}H]Ins(1,3,4,5)P_4 produced by 3-kinase and presented as percentage of the control which is the amount of [^{3}H]Ins(1,3,4,5)P_4 produced in the absence of any synthetic inositol phosphates. Results are shown as the mean ± standard error from three independent experiments.

rational design of selective agonists and antagonists of the Ins(1,4,5)P_3 receptor.

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