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Synthetic inositol 1,3,4,5-tetrakisphosphate analogs and their effect on the binding to microsomal fraction of rat cerebellum

Yuichi Kimura [1], Takashi Kanematsu [1], Yutaka Watanabe [2], Shoichiro Ozaki [2], Toshitaka Koga [1] and Masato Hirata [1]

[1] *Department of Biochemistry, Faculty of Dentistry, Kyushu University, Fukuoka (Japan) and* [2] *Department of Applied Chemistry, Faculty of Engineering, Ehime University, Matsuyama (Japan)*

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Binding activity of [^{3}H]inositol 1,3,4,5-tetrakisphosphate ($InsP_4$) was characterized with rat cerebellar membranes. Two types of $InsP_4$ analog with either the aminobenzoyl or the aminocyclohexanecarbonyl group on the 2nd position of $InsP_4$ have been synthesized and their effects on the binding activity were also examined. [^{3}H]$InsP_4$ binding was gradually displaced by increasing amounts of unlabeled $InsP_4$, with an IC_{50} of 60–170 nM, depending on the pH values. The binding was sharply increased at acidic pH and millimolar concentrations of Ca^{2+}, this being in clear contrast with [^{3}H]$InsP_3$ binding noted in the same species of tissue. Heparin inhibited the binding, with an IC_{50} of 1.7, 3 or 20 μg/ml at pH 8.3, 7.2 or 5.0, respectively. Adenine nucleotide inhibited the binding more potently than did [^{3}H]$InsP_3$ binding. $InsP_4$ analogs were as effective as $InsP_4$ in displacing [^{3}H]$InsP_4$ from rat cerebellar membranes, thereby indicating that the 2nd hydroxl group may not be involved in recognition of $InsP_4$ by its binding sites.

Introduction

Inositol 1,4,5-trisphosphate ($InsP_3$), a product from the hydrolysis of phosphatidylinositol 4,5-bisphosphate by phospholipase C, is well known to cause Ca^{2+} release from intracellular Ca^{2+} store site(s) [1]. $InsP_3$ is phophorylated at position 3 by a specific $InsP_3$ 3-kinase to yield inositol 1,3,4,5-tetrakisphosphate ($InsP_4$) [2]. Irvine and Moor [3] reported that microinjection of $InsP_4$ into sea urchin eggs caused the fertilization envelope, depending on both the presence of extracellular Ca^{2+} and co-injection with a Ca^{2+}-mobilizing compound, inositol 2,4,5-trisphosphate. They suggested that $InsP_4$ may control cellular Ca^{2+} homeostasis through the plasma membrane. Higashida and Brown [4] reported that injection of $InsP_4$ into neuroblastoma × glioma NG108-15 hybrid cells evoked an inward current with a reversal potential of about −20 mV, thereby indicating that $InsP_4$ increased the non-specific cation conductance. In addition, $InsP_4$ is reported to be the activator for the K^+ channel with $InsP_3$ in mouse lacrimal acinar cells [5], protein phosphatase with $InsP_3$ in the rat brain [6], accumulation of Ca^{2+} into Ca^{2+} store site(s) in rat liver cells or cerebellum [7,8], or release of Ca^{2+} from the microsome in the rat cerebellum [9]. Thus, a genenal consensus concerning the function of $InsP_4$ has not been reached. But it was reported that specific binding site(s) for $InsP_4$ are present in the microsomal fraction [10,11], hence, $InsP_4$ is expected to exert its effects on Ca^{2+} homeostasis through the specific binding site(s).

Recently, we synthesized a series of $InsP_3$ analogs, in which the bulky substituent such as 4-azidobenzoyl (designated as analog 195), 4-(5-benzamidoethyl-2-hydroxy-phenylazo)benzoyl (204), 4-aminocyclohexanecarboyl (206) or 4-aminobenzoyl group (209) are coupled with the 2-hydroxyl group of $InsP_3$. Using these analogs, we found that such modifications reduced little the ability of the analogs to interact with receptors and with metabolic enzymes [12,13]. We have now synthesized two types of $InsP_4$ analog which correspond to $InsP_3$ analogs of 206 and 209 [14], and their

Abbreviations: $InsP_4$, inositol 1,3,4,5-tetrakisphosphate; $InsP_3$, inositol 1,4,5-trisphosphate; BPG, bisphosphoglyceric acid; PP_i, pyrophosphate.

Correspondence: M. Hirata, Department of Biochemistry, Faculty of Dentistry, Kyushu University, Fukuoka 812, Japan.

effects on [^{3}H]$InsP_4$-binding was examined. As little is known of the characteristics of the $InsP_4$-binding activity, we also examined this point before attending to the effects of $InsP_4$ analogs. The effects of $InsP_4$ analogs on the phosphatase activities were already described [14].

Materials and Methods

Materials

[^{3}H]$InsP_3$ (specific radioactivity, 754.8 GBq/mmol) and [^{3}H]$InsP_4$ (specific radioactivity, 629 GBq/mmol) were obtained from DuPont-New England Nuclear. D-$InsP_3$ and DL-$InsP_4$ were chemically synthesized, as described [15,16]. D-$InsP_4$ was obtained from Calbiochem. All other reagents were of the highest grade available.

Assay for [^{3}H]$InsP_4$ binding activity of rat cerebellar membranes

Rat cerebellums were homogenized in a glass homogenizer with a Teflon pestle in a solution containing 50 mM Mes buffer (pH 5.0) and 1 mM EDTA. The homogenate was centrifuged at $1500 \times g$ for 10 min, followed by centrifugation at $40000 \times g$ for 20 min and washed once with the same buffer. 50 mM Hepes buffer or Tris-HCl buffer was also used in the place of Mes buffer, depending on the experiments. The washed pellet was suspended in the same buffer and assayed for [^{3}H]$InsP_4$ binding as follows: the mixture (0.5 ml) contained 50 mM pH-buffer (Mes, Hepes or Tris-HCl), 1 mM EDTA, 1.18 nM [^{3}H]$InsP_4$ and approx. 400 μg membrane fraction and incubated on ice for 15 min, followed by passing the mixture through a glass-fiber filter (Whatman GF/C), under vacuum. The filter was washed with 2 ml of the buffer, dried and counted for radioactivity. The protein concentration was determined by the method of Lowry et al. [17] using bovine serum albumin as a standard.

Chemical synthesis of $InsP_4$ analogs (See Fig. 4 for structural formulae)

(i) 2-*O*-(4-Aminobenzoyl)-*myo*-inositol-1,3,4,5-tetraphosphate (KG-194): a solution of 2-*O*-(4'-nitrobenzoyl)-6-benzyl-1,3,4,5-tetrakis (1'-5'-dihydrobenzodioxaphosphelynyl)*myo*-inositol (271 mg) (Ozaki, S., Watanabe, Y. and Koga, Y., unpublished data) and 5% paladium-carbon (Pd/C) (20 mg) in 10 ml of methanol/water (4:1) (v/v) was stirred under hydrogen gas for 24 h. After filtration of the mixture to remove Pd/C, the filtrate was evaporated and chromatographed using an Avicel column (CC-31) to afford KG-194 (79.9 mg, 50%).

(ii) 2-*O*-(4-Aminocyclohexanecarbonyl)*myo*-inositol-1,3,4,5-tetraphosphate (KG-210): a mixture of KG-194 (14.9 mg, 0.0221 mmol) and rutenium oxide (21.5 mg, 0.162 mmol) in water (3.75 ml) and hydrogen gas (80 atm) were charged in a 50 ml autoclave and heated at 60 °C for 2 h. The catalyst was filtered and the filtrate evaporated. The residue was chromatographed using a cellulose column and eluents (*n*-PrOH/conc. NH_3 aq./H_2O, 5:4:1) to give KG-210 (14.3 mg, 96.8%). R_F: 0.24 (*n*-PrOH/conc NH_3 aq./H_2O, 5:5:1).

Results

Characteristics of [^{3}H]$InsP_4$ binding

Rat cerebellum membrane fractions were used for the assay of [^{3}H]$InsP_4$ binding. Binding of [^{3}H]$InsP_4$ (at 1.18 nM) to the membrane fraction was dose-dependently displaced by unlabelled $InsP_4$ (Fig. 1). A 50% displacement of the labelled ligand was attained with 0.06 μM–0.17 μM, depending on the pH values. The binding was specific for $InsP_4$, since $InsP_3$ at 1 or 10 μM displaced the binding by 23% or 38%, respectively, and inositol 1,3,4-trisphosphate was less potent (data not shown).

The specific binding activity was extremely sensitive to pH values as first described for porcine cerebellum by Donié et al. [18,19] (Fig. 2A), but the sensitivity was opposite that seen for [^{3}H]$InsP_3$ binding [20], i.e., an alkaline pH favored the increased binding of [^{3}H]$InsP_3$ [20]. Furthermore, the same phenomenon was observed for the Ca^{2+} dependence of [^{3}H]$InsP_4$ binding (Fig. 2B). The specific binding at both pH 5.0 and 7.2 was increased by 3- to 5-fold by raising Ca^{2+} concentrations to 1 mM, the dependence of which also differed from that for [^{3}H]$InsP_3$ binding [20]. Heparin was also effective in inhibiting the specific [^{3}H]$InsP_4$ binding (Fig. 2C), as was the case with [^{3}H]$InsP_3$ binding first reported by Worley et al. [20]. The concentrations

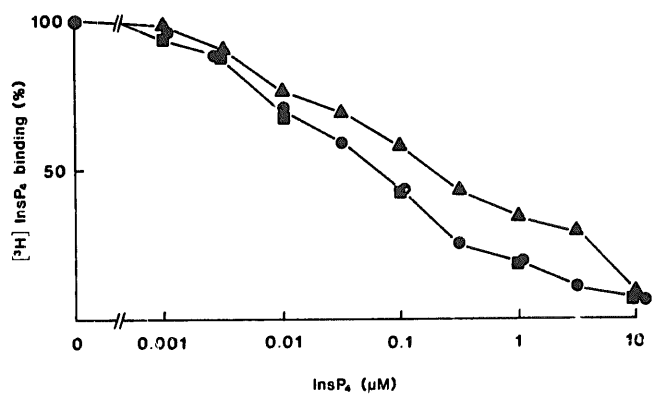

Fig. 1. Displacement of [^{3}H]$InsP_4$ from rat cerebellar membranes by $InsP_4$ at different pH values. Rat cerebellum microsomes were incubated with 1.2 nM [^{3}H]$InsP_4$ in the presence of various concentrations of unlabeled $InsP_4$ on ice for 15 min, at pH 8.3 (■), 7.2 (●) or 5.0 (▲), which were made with Tris-HCl, Hepes or Mes buffer, respectively. Non-specific binding was determined in the presence of 30 μM unlabeled $InsP_4$, and was subtracted from the total binding in its absence to determine the specific binding. Each point is the mean of six, two or eight determinations at pH 8.3, 7.2 or 5.0, respectively.

required for the half-maximal inhibition (IC_{50}) varied, depending on the pH values, i.e., 1.7, 3 and 20 μg/ml at pH 8.3, 7.2 and 5.0, respectively.

Effects of compounds containing the phosphorus moiety on the specific $InsP_4$ binding were then examined, as compared with those of [^{3}H]$InsP_3$ binding to the same tissue. As shown in Fig. 3A, ATP at concentrations ranging from 10–1000 μM, inhibited the [^{3}H]$InsP_4$ binding with the IC_{50} of 35 μM, whereas [^{3}H]$InsP_3$ binding with the IC_{50} of 1000 μM was less sensitive. Although the assay conditions differed in pH value (5.0 vs. 8.3), this would not cause such a different sensitivity to ATP since the dose-dependence in the binding of [^{3}H]$InsP_4$ at pH 7.2 was much the same as that at pH 5.0. GTP and ATP were equipotent in inhibiting [^{3}H]$InsP_4$ binding, while ADP and AMP were less potent, in this order, and adenosine up to 1 mM was ineffective (data not shown), thereby indicating that the phosphorus moiety is apparently responsible for the inhibition. Bisphosphoglyceric acid (BPG) range 0.1–10 mM, and pyrophosphate (PP_i) range 1–100 mM were effective in inhibiting the [^{3}H]$InsP_4$ binding, but in this case, the potency in inhibition was much the same as that seen with [^{3}H]$InsP_3$ binding (Fig. 3B).

Recognition of $InsP_4$ analogs by the binding sites

Two types of $InsP_4$ analog were synthesized. The structure of the compounds are shown in Fig. 4. Whether or not the synthetic $InsP_4$ analogs would compete with [^{3}H]$InsP_4$ for the binding sites was examined using rat cerebellum membranes. As shown in

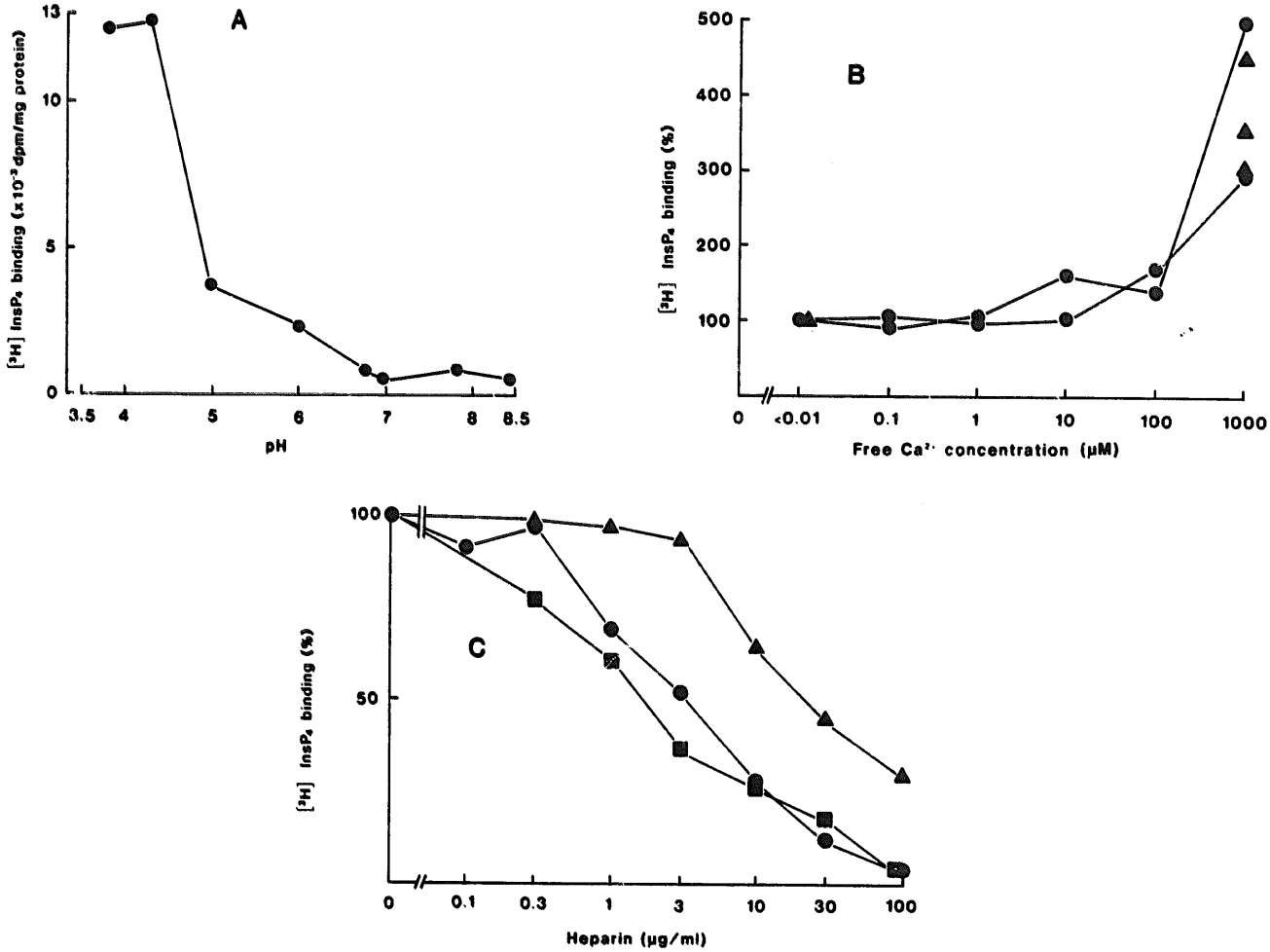

Fig. 2. Dependence of [^{3}H]$InsP_4$ binding on pH (A), free Ca^{2+} concentration (B) and heparin (C). Rat cerebellum microsomes were incubated with 1.2 nM [^{3}H]$InsP_4$ on ice for 15 min. Non-specific binding was determined in the presence of 30 μM unlabeled $InsP_4$, and was subtracted from the total binding in its absence to determine the specific binding. (A) pH-dependence. The specific binding was determined in 50 mM Mes buffer (pH 3.5–6), Hepes buffer (pH 6–8) or Tris-HCl buffer (pH 8–8.5). Each point is the mean of two determinations. (B) Ca^{2+}-dependence. The specific binding was determined at various free Ca^{2+} concentrations at pH 7.2 (●) or 5.0 (▲). In this case, EDTA concentration was reduced to 10 μM and EGTA at 2 mM was included. $CaCl_2$ was added to obtain the desired free Ca^{2+} concentration, under the assumption that the apparent affinity constant of EGTA for Ca^{2+} is $6.3 \cdot 10^6$ M^{-1} at pH 7.2. At pH 5.0, two determinations were made at 0 or 3 mM $CaCl_2$ in the presence of 2 mM EGTA, thus free Ca^{2+} concentration was not calculated. Each point is the mean of duplicate determinations. (C) Heparin-dependence. The specific binding was determined in the presence of various doses of heparin at pH 8.3 (■), 7.2 (●) or 5.0 (▲). Each point is the mean of three determinations.

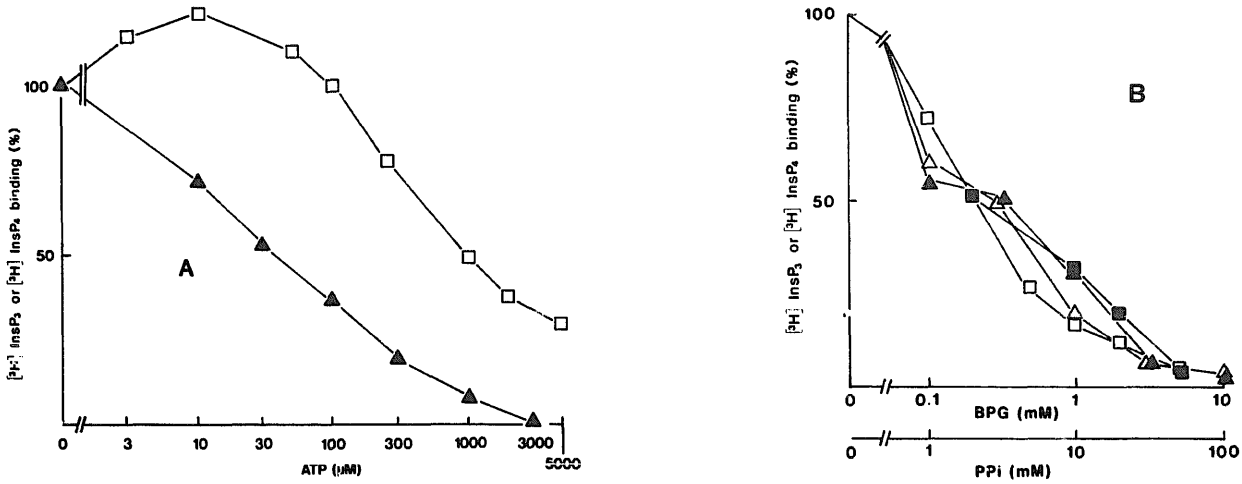

Fig. 3. Effects of ATP, BPG or PP_i on [^{3}H]$InsP_4$ or [^{3}H]$InsP_3$ binding. Rat cerebellum microsomes were incubated with either 1.2 nM [^{3}H]$InsP_4$ at pH 5.0 or 0.98 nM [^{3}H]$InsP_3$ at pH 8.3 on ice for 15 min. Non-specific binding was determined in the presence of 30 μM $InsP_4$ or 1 μM $InsP_3$, and subtracted from the total binding in its absence to determine the specific binding. (A) ATP-effect. Similar inhibition by ATP was also observed at pH 7.2. Each point is the mean of four determinations. (B) BPG- or PP_i-effect. Open symbols, BPG; closed symbols, PP_i. △, ▲: for [^{3}H]$InsP_4$ binding. □, ■: for $InsP_3$ binding. Each point is the mean of two determinations.

Fig. 5, the $InsP_4$ analogs were capable of inducing a dose-dependent displacement of [^{3}H]$InsP_4$ from binding site(s). The potency in the displacement of KG194 was much the same as that of $InsP_4$, while KG210 was slightly less potent.

Discussion

We characterized [^{3}H]$InsP_4$ binding in rat cerebellar membranes, the sites of which may be involved in function(s) not yet been well-defined.

The effects of pH and Ca^{2+} which were opposite to those seen with the [^{3}H]$InsP_3$ binding, do not appear to be physiological relevant as they occur extremely deviated from physiological conditions. The sensitivity to ATP of the binding was greater than that of [^{3}H]$InsP_3$ binding. In contrast, the sensitivities of [^{3}H]$InsP_4$ binding to BPG, PP_i and heparin were equal to those of [^{3}H]$InsP_3$ binding. The greater sensitivity to ATP and GTP, but not to BPG or PP_i may be due to the alignment of three, but not two, phosphorus moieties. However, the observation that ADP also inhibited the [^{3}H]$InsP_4$ binding more potently than did [^{3}H]$InsP_3$ binding would make this proposal unlikely. Thus, adenosine or guanosine moieties in ATP or GTP, albeit ineffective by itself may also be responsible for the greater sensitivity. Theibert et al. [10] reported that ATP inhibited the [^{3}H]$InsP_4$ binding to rat cerebellar membranes, with the IC_{50} of 30 μM at pH 8.5, that is the same as observed here at pH 5.0. On the other hand, Enyedi and Williams [21] reported that ATP at 100 μM or 1 mM only inhibited the [^{3}H]$InsP_4$ binding to calf adrenal cortex membranes by 17% or 72%, respectively. Thus, the binding to peripheral tissues seems to be less sensitive to ATP.

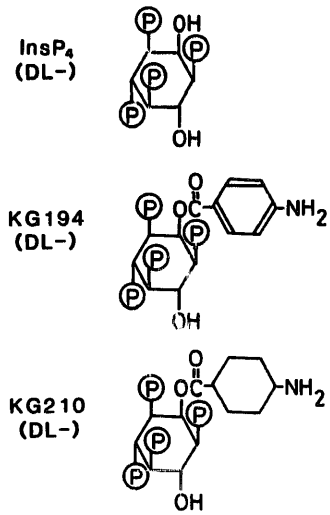

Fig. 4. Structures of synthetic $InsP_4$ analogs. The compounds are a racemic mixture.

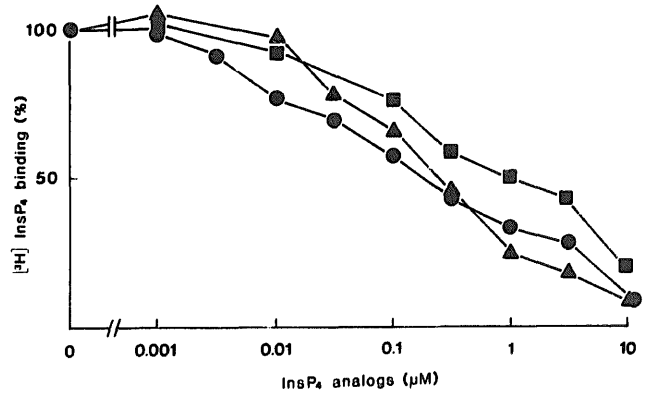

Fig. 5. Displacement of [^{3}H]$InsP_4$ by $InsP_4$ analogs. The specific binding was determined in the presence of various doses of $InsP_4$ analogs at pH 5.0. ▲, KG194; ■, KG210; ●, $InsP_4$. Each point is the mean of five or seven determinations for KG194 or KG210, respectively. Data with $InsP_4$ were re-plotted from Fig. 1. Similar results were obtained at pH 8.3.

The potencies of heparin and $InsP_4$ in inhibiting [^{3}H]$InsP_4$ binding vary, depending on the pH values, i.e., a lower pH decreased the potencies. Change in electrical charges at a different pH in both proteins and inhibitors (heparin and $InsP_4$) can explain such differences. However, ATP and GTP, though their phosphorus moieties were required, were equally effective, regardless of the difference in pH values. We have no reasonable explanation on this point.

$InsP_4$ analogs with either an aminobenzoyl or an aminocyclohexanecarbonyl group on position 2 were capable of inhibiting the binding of [^{3}H]$InsP_4$, with almost the same potencies as $InsP_4$. The result indicates that the 2-hydroxyl group may not be involved in the recognition by the binding sites, and furthermore bulky moieties such as a benzene or a cyclohexane ring on position 2 of $InsP_4$ would not sterically interfere with the recognition. Thus, the results suggest that the $InsP_4$ analogs may be linked to other molecules without loss of their biological activities through the substituent on the 2-position of $InsP_4$. Such a notion is being undertaken, by making $InsP_4$ affinity resins.

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