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MOLECULAR INTERACTIONS OF ALL POSSIBLE REGIOISOMERS OF SYNTHETIC *MYO*-INOSITOL PHOSPHATES WITH INOSITOL 1,4,5-TRISPHOSPHATE 3-KINASE

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Abstract: Inositol 1,4,5-trisphosphate 3-kinase (IP3K) catalyzes the ATP-dependent phosphorylation of inositol 1,4,5-trisphosphate [Ins(1,4,5)P_3] generating inositol 1,3,4,5-tetrakisphosphate [Ins(1,3,4,5)P_4]. The inhibitory effects of all possible 38 regioisomers of inositol phosphates [InsPn] on the IP3K activity have been examined. The correlations between inhibitory potencies and their structural features allowed the assessment of the environment in the active site of IP3K, thus resulting in a proposed binding site model. © 1997 Elsevier Science Ltd.

D-*myo*-Inositol 1,4,5-trisphosphate [Ins(1,4,5)P_3] is a second messenger generated upon hydrolysis of phosphatidylinositol 4,5-bisphosphate in the membrane by a receptor-linked phospholipase C. Ins(1,4,5)P_3 triggers Ca^{2+} release from non-mitochondrial intracellular stores such as endoplasmic reticulum.[1] For its metabolism, Ins(1,4,5)P_3 is first dephosphorylated by Ins(1,4,5)P_3 5-phosphatase (5-phosphatase) to yield Ins(1,4)P_2, or phosphorylated by Ins(1,4,5)P_3 3-kinase (IP3K) to generate Ins(1,3,4,5)P_4.[2] There are increasing evidences that Ins(1,3,4,5)P_4 also serves as a second messenger in the regulation of intracellular calcium concentration.[1,3]

The molecular level characterization of IP3K in catalyzing the phosphorylation of Ins(1,4,5)P_3 has many implications in delineating the complex inositol phosphate metabolism and the Ca^{2+} release mechanism. IP3K shows a remarkable stereo- and regio-selectivity towards the recognition of Ins(1,4,5)P_3, which appears to be higher than that of either Ins(1,4,5)P_3-receptor or 5-phosphatase.[4] For example, Ins(2,4,5)P_3 in which one phosphate group of Ins(1,4,5)P_3 is transposed from C1 to C2, is virtually not recognized by IP3K, while it can bind well to the receptor and to some extent to the 5-phosphatase.[5] For the possible development of either inhibitors or affinity matrix for IP3K, it would be very useful to obtain more detailed information on the binding domain of the enzyme.

Recently, we have systematically synthesized all the possible regioisomers of inositol phosphates from inositol monophosphates ($InsP_1$s) to inositol pentakisphosphates ($InsP_5$s) by the acyl migration method,[6] and

utilized them as molecular probes in the studies of IP_3[7] and IP_4[8] binding proteins. We describe herein the inhibitory effects of all the possible regioisomers of synthetic inositol phosphates on the activity of IP3K, which is purified to near homogeneity.

We have constructed recombinant plasmids for the rat brain IP3K, overexpressed it in *E. coli*, and purified the enzyme to near homogeneity.[9] Then we have examined the inhibitory effects[10] of all the possible 38 regiosomers of synthetic inositol phosphates on the IP3K activity and the results are shown in Table 1.

Several observations stand out from the results. First, no regioisomer of inositol monophosphates, inositol bisphosphates and inositol pentakisphosphates was found effective in inhibiting the IP3K activity even at 100 μM, the highest concentration tested. Second, among the inositol trisphosphate isomers, D/L-Ins(1,4,5)P_3 and D/L-Ins(1,4,6)P_3 significantly inhibited the phosphorylation of D-[^{3}H]-Ins(1,4,5)P_3 by IP3K, D/L-Ins(1,4,6)P_3 (IC_{50}=12.4±3.1 μM) being the most potent inhibitor among the synthetic regioisomers except Ins(1,4,5)P_3, a natural substrate of IP3K. Third, three inositol tetrakisphosphates, D/L-Ins(1,2,4,6)P_4, Ins(1,2,3,5)P_4 and Ins(2,4,5,6)P_4, also exhibited more potent inhibitory activities than D/L-Ins(1,3,4,5)P_4 (IC_{50}=220±48 μM), the product of IP3K. L-Ins(1,4,5)P_3 was previously known to be ca. 50-100 fold less potent than D-Ins(1,4,5)P_3 in the inhibition of IP3K activity.[5,11] Therefore, it is quite clear that the recognition of *myo*-inositol phosphate regioisomers by IP3K is highly structure-selective.

Very weak inhibition by inositol monophosphates and inositol bisphosphates indicates that all three phosphate groups of D-Ins(1,4,5)P_3 are essential for the efficient binding to IP3K. Even though D-Ins(1,3,6)P_3 has similar dispositions of the three phosphate groups as D-Ins(1,4,5)P_3 (**1a** vs. **1d**), it does not inhibit the IP3K activity as was previously reported.[12] The lack of the inhibitory activity is likely due to the inverted stereochemistry at 2-OH and 4-OH of D-Ins(1,3,6)P_3 compared to D-Ins(1,4,5)P_3. D-Ins(1,4,6)P_3 with properly oriented three phosphates and the equatorial hydroxyl group at the C-5 position, which is equivalent to the 6-OH of D-Ins(1,4,5)P_3, efficiently inhibited the IP3K activity despite the inverted 2-OH and 3-OH groups, suggesting that the configurations at 2-OH and 3-OH of D-Ins(1,4,5)P_3 may not be critical to the binding to IP3K (**1a** vs. **1c**). The major structural features responsible for the inhibitory action of D-Ins(1,4,6)P_3 might be the proper equatorial hydroxyl group at C-5 position in addition to the three phosphate groups. Regarding the equatorial OH group at the C-6 position of D-Ins(1,4,5)P_3, the bulky phosphate group present in D-Ins(1,4,5,6)P_4, **1e** appears to hinder its binding to IP3K, rendering the IC_{50} value of D/L-Ins(1,4,5,6)P_4 higher than that of D/L-Ins(1,3,4,5)P_4, a very weak inhibitor of IP3K.[4b] From these observations, it is fairly obvious that the 1,4,5-trisphosphate motif of D-Ins(1,4,5)P_3 is of primary importance, and all subsequent comparisons of the inhibition data are based on this premise (vide infra). It is evident that the steric factor of the substituent at the C-6 position of D-Ins(1,4,5)P_3 is very important for binding to IP3K, since either the stereoinversion or a bulky substituent at this position is not tolerated.

Table 1. Inhibition of IP3K by Synthetic Inositol Phosphate Regioisomers.

Inositol Phosphates	$IC_{50}(\mu M)$
D/L-Ins(1,4,5)P_3	3.55±0.11
D/L-Ins(1,4,6)P_3	12.4±3.1
D/L-Ins(1,2,4,6)P_4	42.1±3.2
Ins(1,2,3,5)P_4	65.2±4.3
Ins(2,4,5,6)P_4	56.7±14.3
D/L-Ins(1,3,4,5)P_4	220±48

IC_{50} values were presented for the regioisomers which exhibited inhibition effect on IP3K activity better than Ins(1,3,4,5)P_4, a very weak inhibitor of IP3K.

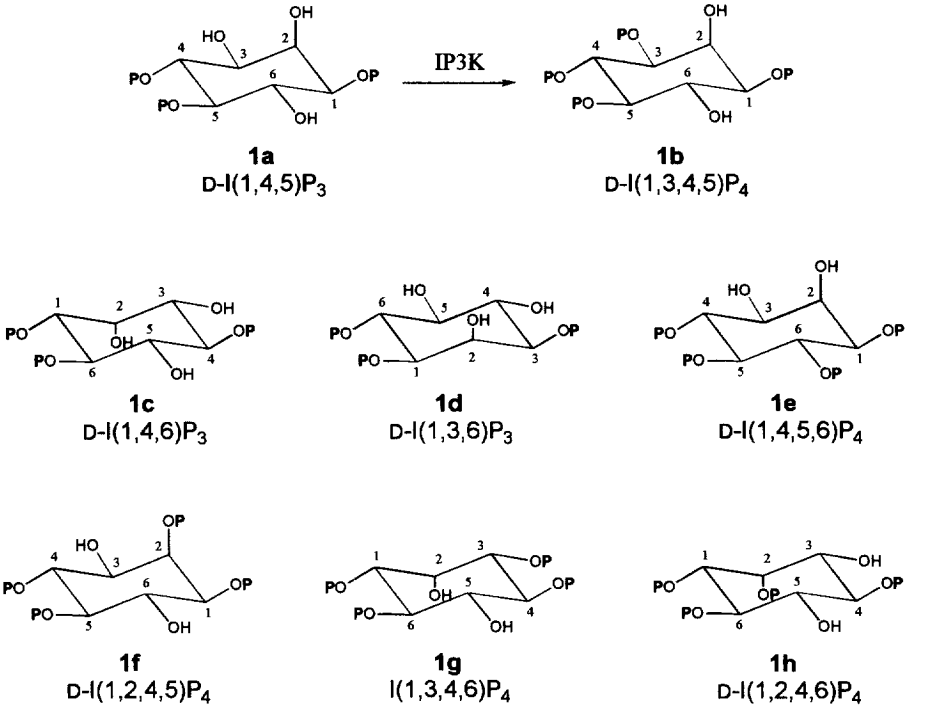

Fig.1. Structures of important regioisomers among synthetic inositol phosphates. All these regioisomers used in the experiments were D/L-mixtures except Ins(1,3,4,6)P_4 which is a meso compound. Only the D-isomers of the inositol phosphates are illustrated in the figure.

Although the 2-OH of D-Ins(1,4,5)P_3 is not critically important for its binding to IP3K,[12,13] introduction of a bulky phosphate group at the 2-position or at its equivalent positions in such regioisomers as D/L-Ins(1,2,4,5)P_4 and D/L-Ins(1,3,4,6)P_4 (**1f**, **1g** and Fig.2) was found to hinder the binding in accord with the previous observations that these compounds weakly inhibit the IP3K activity.[11,14] Based on this observation and the report that bulky neutral substituents such as benzoyl or methylbenzoyl groups at the C-2 position are well tolerated by IP3K,[15] IP3K seems to have a vacant space at the C-2 axial direction of D-Ins(1,4,5)P_3 and the binding site of IP3K appears to have negative charges near the vacant space surrounding the C-2 position of D-Ins(1,4,5)P_3. The proposed negatively-charged pocket causes unfavorable interactions between IP3K and the 2-phosphate group of D/L-Ins(1,2,4,5)P_4.

The inhibition of the IP3K activity by the IP_n regioisomers was found to vary depending upon the stereochemistry of 3-OH. For example, D-Ins(1,2,4,6)P_4 and D-Ins(1,3,4,5)P_4 (**1h** vs. **1b**, Fig. 2) are equivalent in terms of the 1,4,5-trisphosphate motif, but differ in the stereochemical orientations of the C-3 phosphate and C-2 hydroxyl group with reference to D-Ins(1,4,5)P_3. D-Ins(1,4,5)P_3 derivatives with a bulky substituent such as 3-benzoyl- or 3-methylbenzoyl group are ineffective in inhibiting the IP3K activity, implying that IP3K does not tolerate a bulky substituent at that position.[15] The 2-phosphate group of Ins(1,2,4,6)P_4 corresponds to the 3-phosphate of Ins(1,3,4,5)P_4 with its axially inverted orientation as shown in **1h** and **1b**. It is interesting to note that D/L-Ins(1,2,4,6)P_4 shows a substantially higher inhibitory activity than Ins(1,3,4,5)P_4. Thus, IP3K seems to have a limited space in the C-3 equatorial direction of D-Ins(1,4,5)P_3, while it has in the axial direction a vacant space large enough to accommodate the bulky phosphate group. It is speculated that this empty space of IP3K may be occupied by ATP.

It is somewhat surprising that both Ins(1,2,3,5)P_4 and Ins(2,4,5,6)P_4 with rather unusual patterns of the phosphate groups in the inositol ring can be found among the regioisomers of the inositol phosphates that exhibit meaningful inhibition activities for IP3K. Neither of these inositol tetrakisphosphates has the presumed structural requirement of the proper dispositions of phosphate groups, i.e. the D-1,4,5-trisphosphate motif. It can only be speculated that the available vacant spaces of IP3K near the C-2 and C-3 positions of D-Ins(1,4,5)P_3 may play a role in accommodating these unusual inositol phosphates in its binding pocket.

Based on the correlations between the distinct stereochemical disposition of inositol phosphate regioisomers with a meaningful inhibitory potency and their binding affinity to IP3K, we propose a binding site model for IP3K (Fig. 3). The binding site environment, especially the locations of the vacant spaces may be exploited for the design of useful derivatives and analogs of D-Ins(1,4,5)P_3. Furthermore, understanding the key binding site structure differences between IP3K, 5-phosphatase and IP_3 receptors may be critically useful in developing selective receptor antagonists or enzyme inhibitors for pharmacological applications.

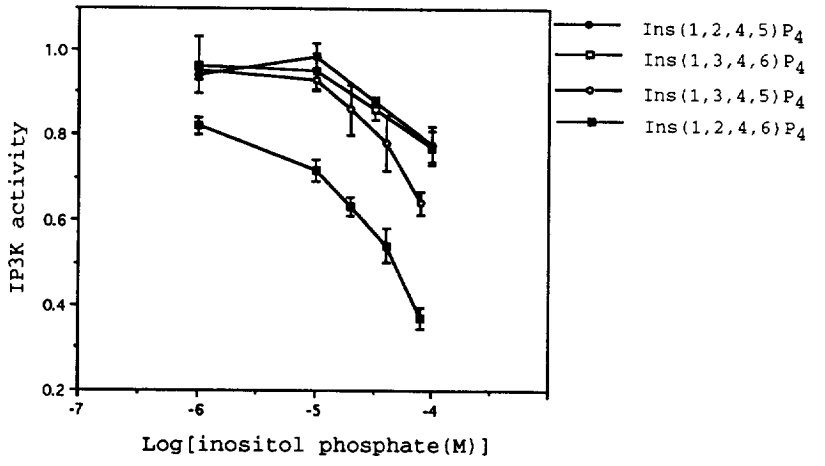

Fig.2. Inhibition of IP3K activity by inositol tetrakisphosphates which have a phosphate group at either C-2 or C-3 position of D-Ins(1,4,5)P_3. □: D/L-Ins(1,3,4,6)P_4 ■: D/L-Ins(1,2,4,6)P_4 ○: D/L-Ins(1,3,4,5)P_4 ●: D/L-Ins(1,2,4,5)P_4. Assays were performed with 1 μM [^{3}H]-D-Ins(1,4,5)P_3 by increasing the concentration of each inositol phosphate in the absence of Ca^{2+}. The IP3K activity represents the relative value to that in the absence of any inhibitior. Results are shown as the mean ± standard deviation from three independent experiments.

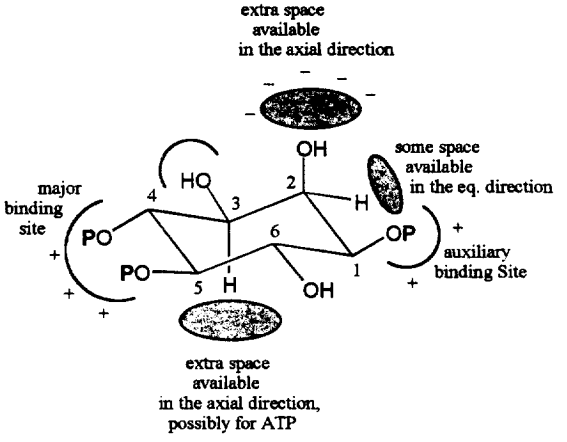

Fig.3. Proposed binding model of IP3K for Ins(1,4,5)P_3.

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