Notes

SCREENING OF PHOSPHATIDYL-INOSITOL KINASE INHIBITORS FROM *STREPTOMYCES*

Hiroshi Nishioka, Masaya Imoto, Tsutomu Sawa, Masa Hamada, Hiroshi Naganawa, Tomio Takeuchi and Kazuo Umezawa

Institute of Microbial Chemistry, 3-14-23 Kamiosaki, Shinagawa-ku, Tokyo 141, Japan

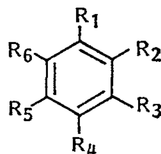
(Received for publication December 22, 1988)

Phosphatidylinositol turnover is important in intracellular signal transduction in response to various growth factors, hormones and neurotransmitters[1]. Transformation by oncogenes such as *ras*[2], *src*[3], *erbB*[4], *fms*[5] and *fes*[5] also enhances cellular phosphatidylinositol turnover. Phosphatidylinositol kinase is an enzyme involved in the pathway of phosphatidylinositol turnover. Especially, its activity is known to be enhanced by transformation with *src*[3,6], *erbB*[4], *fms*[6] and *sis*[6] and by platelet-derived growth factor[6]. Therefore, we have screened for inhibitors of phosphatidylinositol kinase from microbial secondary metabolites and found that 2,3-dihydroxybenzoic acid and orobol strongly inhibit the enzyme.

The phosphatidylinositol kinase from A431 cells has been purified to near homogeneity[7]. The enzyme has a subunit weight of 55,000 and acts as a monomer. It phosphorylates the inositol moiety of phosphatidylinositol on the 4-position and is therefore phosphatidylinositol 4-kinase (Type II)[8].

With the A431 cell membrane, we have developed a rapid assay method for phosphatidylinositol kinase using a small packed silica gel column for separating phosphorylated lipid and unreacted [γ-^{32}P]ATP. The reaction mixture of phosphatidylinositol (60 μg) and membrane fraction of A431 cells with or without inhibitor was incubated in 90 μl of 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer (pH 7.2) at 20°C for 10 minutes, then [γ-^{32}P]ATP was added to give about 2 μM final concentration and incubation was continued at 20°C for 20 minutes. The reaction was stopped by addition of 700 μl of a mixture of $CHCl_3$, MeOH and 1 N HCl (4:1:2). The mixture was centrifuged (3,000 rpm × 10 minutes) to separate the two phases. Then, 200 μl of the lower phase was

Table 1 Inhibition of phosphatidylinositol (PI) kinase by benzoic acids and benzaldehydes.



R_1	R_2	R_3	R_4	R_5	R_6	PI kinase IC_{50} (μg/ml)
COOH	OH	OH	H	H	H	0.7
COOH	OH	H	OH	H	H	>10
COOH	OH	H	H	OH	H	>10
COOH	OH	H	H	H	OH	>10
COOH	H	OH	OH	H	H	5.6
COOH	H	OH	H	OH	H	>10
COOH	OH	H	H	H	H	>10
CHO	OH	OH	H	H	H	0.45
CHO	OH	H	OH	H	H	>10
CHO	H	OH	OH	H	H	10
CHO	OH	OH	OH	H	H	>10

applied to a packed silica gel column (1 ml). The phosphorylated lipid was eluted with a mixture of $CHCl_3$, MeOH and 4 N NH_4OH (9 : 7 : 2) and the eluted fraction was evaporated to dryness *in vacuo*. The residue was dissolved in 100 μl of a mixture of $CHCl_3$ and MeOH (2 : 1). Then, 80 μl of the solution was applied on a cellulose filter paper. The paper was dried and counted for radioactivity.

After screening for phosphatidylinositol kinase inhibitors from hundreds of *Streptomyces* culture filtrates, we isolated 2,3-dihydroxybenzoic acid which inhibited the enzyme with an IC_{50} of 0.7 μg/ml. For isolation of 2,3-dihydroxybenzoic acid, *Streptomyces* strain MI689-SF6 was cultured in media containing glucose 2%, starch 3%, corn steep liquor 1%, soybean meal 0.2%, Polypeptone 0.5%, NaCl 0.3%, $CaCO_3$ 0.5% (pH 7.0) for 4 days at 28°C on a reciprocal shaker. The broth filtrate (3 liters) was extracted with BuOAc (3 liters) after adjusting to pH 2.0. The extract was concentrated *in vacuo* to give an oily matter which was mixed with silica gel (8.5 g) and applied to a silica gel column chromatography (31.5 g). The column was washed with a mixture of $CHCl_3$ and MeOH, and an active compound was eluted with a mixture of BuOH, MeOH and H_2O (4 : 1 : 2). The active fractions were charged on a column of Toyopearl HW-40 (150 ml) which was developed with MeOH. The residue after evaporation of the active fractions was further chromatographed on Sephadex LH-20 (100 ml) with MeOH. Then, the active compound was further purified by planetary coil centrifuge to yield 4.4 mg of 2,3-dihydroxybenzoic acid which was identified by ^1H and ^{13}C NMR and mass spectra.

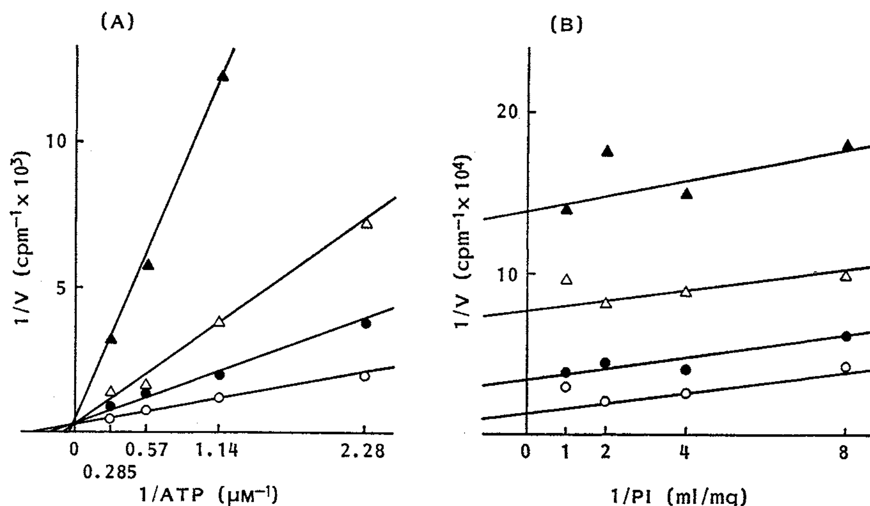
Among its structural analogues 2,4-, 2,5-, 2,6-, 3,4-, 3,5-dihydroxy and *o*-hydroxybenzoic acid were inactive, as shown in Table 1. An aldehyde analogue, 2,3-dihydroxybenzaldehyde showed stronger inhibitory activity with an IC_{50} of 0.45 μg/ml. But, 2,4-, 3,4-dihydroxy and 2,3,4-trihydroxybenzaldehyde did not inhibit the enzyme. Thus, the 2,3-dihydroxy substitution is favorable for the enzyme inhibition. In

Table 2. Inhibition of phosphatidylinositol (PI) kinase by flavonoids.

	PI kinase IC_{50} (μg/ml)
Isoflavones	
Orobol	0.25
Psi-tectorigenin	>10
Genistein	>10
Daizein	>10
Flavones	
Quercetin	1.8
Fisetin	2.0
Galangin	>10
Kaempferol	>10

Fig. 1. Lineweaver-Burk plot of the phosphatidylinositol (PI) kinase reaction with orobol.

The reaction was carried out with 0 μg/ml (○), 0.25 μg/ml (●), 0.5 μg/ml (△) or 1.0 μg/ml (▲) of orobol. (A) Orobol against ATP, (B) orobol against phosphatidylinositol.



addition, 2,3-dihydroxybenzoic acid and aldehyde inhibited tyrosine kinase of epidermal growth factor receptor with IC_{50} of about 10 μg/ml and 1 μg/ml, respectively.

Quercetin is known to inhibit phosphatidylinositol kinase. In our assay system it inhibited the enzyme with an IC_{50} of 1.8 μg/ml. Therefore, we have studied inhibition of phosphatidylinositol kinase by flavonoids which are structurally related to quercetin.

As shown in Table 2, orobol isolated from *Aspergillus niger*[9] or *Streptomyces neyagawaensis*[10] was found to be a potent inhibitor of phosphatidylinositol kinase. Its inhibitory activity was about 10 times stronger than that of quercetin. Orobol also inhibits cellular phosphatidylinositol turnover in A431 cells[11]. Psi-tectorigenin strongly inhibits cellular phosphatidylinositol turnover[11], but it does not inhibit phosphatidylinositol kinase. Thus, it should inhibit other enzymes involved in phosphatidylinositol turnover. Orobol is known to inhibit tyrosine kinase competitively with ATP[12]. Again it was shown to compete with ATP in inhibition of phosphatidylinositol kinase. As shown in Fig. 1, kinetic studies by Lineweaver-Burk plotting have indicated that orobol is competitive with ATP and is uncompetitive with phosphatidylinositol. The Dixon plot of orobol with ATP has shown that the *Ki* value is 0.49 μM.

Acknowledgment

This work was partly supported by Grant-in-Aid for New Drug Development Research and by a Grant-in-Aid for the Comprehensive 10-year Strategy for Cancer Control from the Ministry of Health and Welfare of Japan.

References

1) BERRIDGE, M. J. & R. F. IRVINE: Inositol triphosphate, a novel second messenger in cellular signal transduction. Nature 312: 315~321, 1984
2) FLEISCHMAN, L. F.; S. B. CHAHWALA & L. CANTLEY: *Ras*-transformed cells: Altered levels of phosphatidylinositol-4,5-bisphosphate and catabolites. Science 231: 407~410, 1986
3) SUGIMOTO, Y. & R. L. ERIKSON: Phosphatidylinositol kinase activities in normal and Rous sarcoma virus-transformed cells. Mol. Cell. Biol. 5: 3194~3198, 1985
4) KATO, M.; S. KAWAI & T. TAKENAWA: Altered signal transduction in *erb*B-transformed cells. J. Biol. Chem. 262: 5696~5704, 1987
5) JACKOWSKI, S.; C. W. RETTENMIER, C. J. SHERR & C. O. ROCK: A guanine nucleotide-dependent phosphatidylinositol 4,5-diphosphate phospholipase C in cells transformed by the v-*fms* and v-*fes* oncogenes. J. Biol. Chem. 261: 4978~4985, 1986
6) KAPLAN, D.R.; M. WHITMAN, B. SCHAFFHAUSEN, D. C. PALLAS, M. WHITE, L. CANTLEY & T. M. ROBERTS: Common elements in growth factor stimulation and oncogenic transformation: 85 kd phosphoprotein and phosphatidylinositol kinase activity. Cell 50: 1021~1029, 1987
7) WALKER, D. H.; N. DOUGHERTY & L. J. PIKE: Purification and characterization of a phosphatidylinositol kinase from A431 cells. Biochemistry 27: 6504~6511, 1988
8) WHITMAN, M.; C. P. DOWNES, M. KEELER, T. KELLER & L. CANTLEY: Type I phosphatidylinositol kinase makes a novel inositol phospholipid, phosphatidylinositol-3-phosphate. Nature 332: 644~646, 1988
9) UMEZAWA, H.; H. TOBE, N. SHIBAMOTO, F. NAKAMURA, K. NAKAMURA, M. MATSUZAKI & T. TAKEUCHI: Isolation of isoflavones inhibiting DOPA decarboxylase from fungi and streptomyces. J. Antibiotics 28: 947~952, 1975
10) UMEZAWA, H.; M. IMOTO, T. SAWA, K. ISSHIKI, N. MATSUDA, T. UCHIDA, H. IINUMA, M. HAMADA & T. TAKEUCHI: Studies on a new epidermal growth factor-receptor kinase inhibitor, erbstatin, produced by MH435-hF3. J. Antibiotics 39: 170~173, 1986
11) IMOTO, M.; T. YAMASHITA, T. SAWA, S. KURASAWA, H. NAGANAWA, T. TAKEUCHI, Z. BAO-QUAN & K. UMEZAWA: Inhibition of cellular phosphatidylinositol turnover by psi-tectorigenin. FEBS Lett. 230: 43~46, 1988
12) IMOTO, M.; K. UMEZAWA, K. ISSHIKI, S. KUNIMOTO, T. SAWA, T. TAKEUCHI & H. UMEZAWA: Kinetic studies of tyrosine kinase inhibition by erbstatin. J. Antibiotics 40: 1471~1473, 1987