INHIBITION OF PHOSPHATIDYLINOSITOL KINASE BY TOYOCAMYCIN

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We have screened toyocamycin as an inhibitor of phosphatidylinositol kinase. It inhibited the enzyme of A431 cell membrane with an IC_{50} of 3.3 μg/ml. Adenosine and formycin A also inhibited the enzyme, but other 6 related nucleosides did not. Although orobol and 2,3-dihydroxybenzaldehyde that inhibit phosphatidylinositol kinase inhibited *in situ* phosphatidylinositol turnover, toyocamycin did not.

Phosphatidylinositol turnover is considered to be implicated in the cellular response to mitogens[1] and transformation by oncogenes such as *ras*[2], *src*[3], *erbB*[4], *fms* and *fes*[5]. Phosphatidylinositol kinase is one of the key regulatory enzymes involved in the pathway of phosphatidylinositol turnover. Therefore, we are sceening inhibitors of phosphatidylinositol kinase from microorganisms to study the biochemical role of the enzyme. We have developed a new assay system for phosphatidylinositol kinase using a small silica gel column, and have recently found that orobol and 2,3-dihydroxybenzoic acid from microorganisms inhibit the enzyme[6]. In the course of this screening program, we have isolated toyocamycin from a culture filtrate of a *Streptomyces* strain. Toyocamycin is known as an antifungal antibiotic[7]. This paper reports inhibition of phosphatidylinositol kinase by toyocamycin and other related nucleoside antibiotics. In addition, we have studied effects of toyocamycin, orobol and 2,3-dihydroxybenzaldehyde, a more effective analogue of 2,3-dihydroxybenzoic acid, on *in situ* phosphatidylinositol turnover.

Materials and Methods

Materials

Sangivamycin, tubercidin, formycins A and B, and oxanosine were isolated from microorganisms in the Institute of Microbial Chemistry. SH-formycin (7-deamino-7-mercaptoformycin) was a kind gift from Meiji Seika Kaisha, Ltd. Adenosine and inosine were purchased from Sigma Chemical Company. [γ-^{32}P]ATP and myo-[^{3}H]inositol were purchased from DuPont—New England Nuclear. A431 cell line was kindly supplied by Dr. S. KAWAI, Institute of Medical Science, University of Tokyo.

Isolation of Toyocamycin

The *Streptomyces* strain MI698-50F1 was cultured in media containing glycerol 2.0%, soy bean meal 1.5%, K_2HPO_4 0.1% and $CoCl_2 \cdot 6H_2O$ 0.0005%. The medium was adjusted to pH 6.2 with 1 M KH_2PO_4 prior to the addition of silicon oil as antifoam and the fermentation was allowed to proceed for 8 days at 28°C on a rotary shaker. The broth filtrate (3.6 liters) was extracted with 1-BuOH (3.6 liters). The extract was concentrated to dryness and the residue (3.9 g) was treated with MeOH. The soluble portion was mixed with silica gel (7 g) and applied to a silica gel column (100 g) for chromatography. The column was washed with a mixture of $CHCl_3$ and MeOH (10 : 1) and the active fraction was eluted with a mixture of $CHCl_3$ and MeOH (5 : 1). The crude material (400 mg) was dissolved in a small amount of DMF and

chromatographed on Sephadex LH-20 (200 ml) with MeOH. After concentration of the active fractions to dryness, the residue was crystallized from MeOH to give the purified compound (49 mg). Structure determination and spectroscopic analysis revealed that it was identical to toyocamycin.

Phosphatidylinositol Kinase Assay

Enzyme assay *in vitro* was carried out as described previously[6]. The reaction mixture of phosphatidylinositol, membrane fraction of A431 cells and [γ-^{32}P]ATP with or without inhibitors was incubated in 20 mM 4-2-(hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer (pH 7.2) at 20°C. The reaction was stopped by addition of a mixture of $CHCl_3$, MeOH and 1 N HCl (4 : 1 : 2). After vortexing, the lower phase was taken and applied to a packed silica gel column for separating phosphorylated lipid and unreacted [γ-^{32}P]ATP. Then, phosphorylated lipid was eluted with a mixture of $CHCl_3$, MeOH and 4 N NH_4OH (9 : 7 : 2) and counted for radioactivity.

In situ Phosphatidylinositol Turnover Assay

A431 cells (3×10^5 cells/well) plated on 35-mm plastic wells were grown in DULBECCO's modified EAGLE's medium supplemented with 5% calf serum for 16 hours. The cells were pre-incubated in 1 ml of HEPES-buffered saline (HBS) (HEPES 20 mM, NaCl 150 mM, KCl 5 mM, $CaCl_2$ 1 mM, $MgCl_2$ 1 mM and glucose 0.1%, pH 7.4) containing myo-[^{3}H]inositol (1 μCi/ml) at 37°C for 30 minutes, then, an inhibitor and epidermal growth factor (EGF) (400 ng/ml) were added, and the incubation was continued at 37°C for 60 minutes. The cells were washed with 2 ml of 10% TCA containing 0.01 M sodium pyrophosphate then added by 0.5 ml of 0.5 N NaOH. After removal of the solubilized fraction, the cells remained were scraped off with rubber policeman from the dishes in 1.0 ml of H_2O. They were combined and counted for radioactivity.

Inositol Phosphates Formation

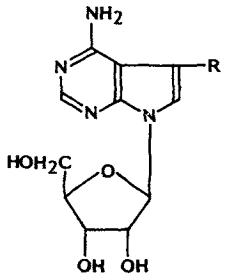
A431 cells (3×10^5 cells/well) plated on 24-mm plastic dishes were grown for 16 hours before use. The cells were labeled with myo-[^{3}H]inositol in 0.5 ml of HBS containing 30 mM LiCl in the presence or absence of test chemical. After addition of 400 ng/ml of EGF, the cells were incubated for further 120 minutes. Then, the incubation was terminated by adding ice-cold 10% $HClO_4$ (100 μl) and the mixture was neutralized by 75 mM HEPES containing 1.53 M KOH. The solution was kept on ice for 15 minutes, and centrifuged at $2,000 \times g$ for 15 minutes at 4°C. The supernatant was applied to anion-exchange column (Amprep SAX, Amersham) and eluted with 0.17 M $KHCO_3$. The sum of the labeled inositol phosphates ($IP + IP_2 + IP_3$) were measured by liquid scintillation counter.

Results and Discussion

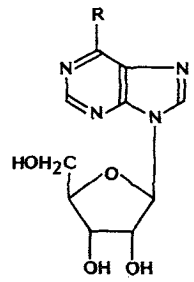
As shown in Fig. 1, toyocamycin inhibited phosphatidylinositol kinase with an IC_{50} of 3.3 μg/ml. The IC_{50}s of adenosine and formycin A were 4.3 μg/ml and 7.0 μg/ml, respectively. Inosine, formycin B and SH-formycin[8] did not inhibit the enzyme (IC_{50}, >100 μg/ml). Sangivamycin is known to inhibit purified rat brain protein kinase C competitively against ATP with an inhibition constant (*Ki*) value of 10 μM[9]. It also inhibits autophosphorylation of EGF receptor of the A431 cell membrane *in vitro*[10]. Although sangivamycin and tubercidin have the same nucleoside skeleton as toyocamycin, they showed only weak inhibitory activities in our assay system (IC_{50}, >40 μg/ml). Inhibition of protein kinase C by toyocamycin was reported to be about 30 times weaker than that by sangivamycin[10]. Inhibition of tyrosine kinase by toyocamycin was also ten times weaker than that by sangivamycin[10]. Oxanosine was reported to inhibit *ras* oncogene functions in rat kidney cells transformed with a temperature-sensitive Kirsten sarcoma virus[11]. It did not inhibit phosphatidylinositol kinase at 100 μg/ml. Thus, these results indicated that toyocamycin is an unique nucleoside antibiotic which inhibits phosphatidylinositol kinase.

As shown in Table 1, orobol and 2,3-dihydroxybenzaldehyde inhibited EGF-stimulated incorporation

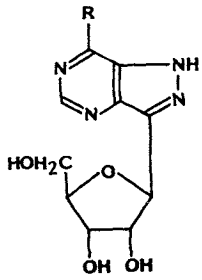
Fig. 1. Inhibition of phosphatidylinositol kinase by nucleosides and analogues.
Values indicate concentrations for 50% inhibition.



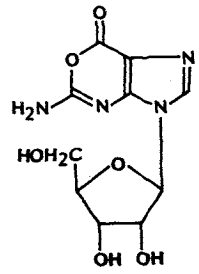
Toyocamycin R=CN 3.3 μg/ml
Sangivamycin R=$CONH_2$ >40 μg/ml
Tubercidin R=H >40 μg/ml



Adenosine R=NH_2 4.3 μg/ml
Inosine R=OH >100 μg/ml



Formycin A R=NH_2 7.0 μg/ml
Formycin B R=OH >100 μg/ml
SH-Formycin R=SH >100 μg/ml



Oxanosine >100 μg/ml

Table 1. Inhibition of phosphatidylinositol (PI) turnover by PI kinase inhibitors.

	PI turnover IC_{50} (μg/ml)
Toyocamycin	>100
Orobol	15
2,3-Dihydroxybenzaldehyde	7.0

of myo-[^{3}H]inositol into phospholipids with IC_{50}s of 15 μg/ml and 7.0 μg/ml, respectively. However, toyocamycin did not affect the incorporation even at 100 μg/ml.

Table 2. Effect of phosphatidylinositol (PI) kinase inhibitors on inositol phosphates formation.

Chemical (10 μg/ml)	[^{3}H]inositol phosphates (dpm)
None	77.21± 8.98
+EGF	1,415.37±102.80
+EGF+Toyocamycin	1,856.28±106.58
+EGF+Orobol	632.46± 88.80*
+EGF+2,3-Dihydroxy-benzaldehyde	580.96± 53.08*

* $P<0.05$.

Effects of phosphatidylinositol kinase inhibitors on EGF-induced inositol phosphates formation are shown in Table 2. Orobol and 2,3-dihydroxybenzaldehyde inhibited inositol phosphates formation at 10 μg/ml, but toyocamycin did not. Since toyocamycin inhibits the growth of A431 cells with an IC_{50} of 2.3 ng/ml, it is considered to penetrate into the cells. Toyocamycin is weaker than orobol (IC_{50}, 0.25 μg/ml) or 2,3-dihydroxybenzaldehyde (IC_{50}, 0.45 μg/ml) in *in vitro* inhibition of phosphatidylinositol kinase. Therefore, higher concentration of toyocamycin might be necessary to inhibit phosphatidylinositol turnover *in situ*. It is also possible that subcellular localization of toyocamycin might be different from those of orobol and 2,3-dihydroxybenzaldehyde.

The A431 cell membrane contains phosphatidylinositol-4-kinase[12] which is involved in phosphatidylinositol turnover. Recently, phosphatidylinositol-3-kinase was found to be associated with platelet-derived growth factor receptor[13,14], colony stimulating factor-1 receptor[15] and insulin receptor[16], and it might be more important than phosphatidylinositol-4-kinase for regulation of cell proliferation. Inhibition of phosphatidylinositol-3-kinase by toyocamycin, orobol and 2,3-dihydroxybenzaldehyde is being studied.

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