Volume 230, number 1,2, 43–46 FEB 05695 March 1988

Inhibition of cellular phosphatidylinositol turnover by psi-tectorigenin

Masaya Imoto, Takashi Yamashita, Tsutomu Sawa, Shogo Kurasawa, Hiroshi Naganawa, Tomio Takeuchi, Z. Bao-quan* and Kazuo Umezawa

Institute of Microbial Chemistry, 3-14-23 Kamiosaki, Shinagawa-ku, Tokyo 141, Japan and **Shanghai Institute of Pharmaceutical Industry, 1320 Beijing Road (W), Shanghai, PR China*

Received 2 February 1988

Psi-tectorigenin, an isoflavonoid, was isolated from a culture filtrate of actinomycetes as an inhibitor of epidermal growth factor-induced phosphatidylinositol turnover in cultured A431 cells. It inhibited phosphatidylinositol turnover with an IC_{50} of about 1 μg/ml; thus, its inhibitory activity was 6-times stronger than that of genistein or orobol. When added to cultured A431 cells psi-tectorigenin inhibited phosphatidylinositol turnover without inhibiting epidermal growth factor receptor tyrosine protein kinase. Thus, psi-tectorigenin is a specific inhibitor of phosphatidylinositol turnover and may be a useful tool for the functional analysis of phosphatidylinositol turnover.

Phosphatidylinositol turnover; Psi-tectorigenin; Tyrosine kinase; (Actinomycete)

1. INTRODUCTION

We have previously isolated erbstatin from a *Streptomyces* culture filtrate as an inhibitor of tyrosine-specific protein kinase [1]. It inhibited epidermal growth factor (EGF)-induced autophosphorylation of EGF receptor and autophosphorylation of the $pp60^{src}$ protein. However, it did not inhibit EGF-induced phosphatidylinositol (PtdIns) turnover in cultured A431 cells [2].

Increased turnover of PtdIns and of its phosphorylated derivatives, phosphatidylinositol 4-phosphate (PtdIns-P) and 4,5-bisphosphate ($PtdIns-P_2$), has been implicated in the cellular response to growth factors such as EGF [3], platelet-derived growth factor [4], or interleukin-2 [5]. $PtdIns-P_2$ breakdown generates two second messengers, diacylglycerol and inositol trisphosphate. Diacylglycerol activates protein kinase C [6], and inositol trisphosphate mobilizes calcium from an internal source [7]. Furthermore, several studies have shown that levels of inositol trisphosphate and diacylglycerol are elevated in a variety of transformed cell lines [8–10]. Taken together, these reports led to the speculation that viral transformation might be initiated through an increase in phosphorylation of PtdIns resulting in an increase in the supply of inositol trisphosphate and diacylglycerol which then would act as second messengers to stimulate cell growth.

Therefore, we have screened culture filtrates of microorganisms for an inhibitor of PtdIns turnover and isolated psi-tectorigenin.

Correspondence address: M. Imoto, Institute of Microbial Chemistry, 3-14-23 Kamiosaki, Shinagawa-ku, Tokyo 141, Japan

2. MATERIALS AND METHODS

2.1. *Materials*

EGF was purchased from Takara Shuzo. [^{3}H]Inositol (14.0 Ci/mmol) and [^{32}P]orthophosphate ($^{32}P_i$) (1 mCi/ml) were obtained from Dupon, while [γ-^{32}P]ATP (10.54 Ci/mmol) was from New England Nuclear. The synthetic peptide RR-SRC was purchased from the Peptide Institute (Osaka). The A431 cell line was obtained from Dr S. Kawai (Institute of Medical Science, University of Tokyo).

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/88/$3.50

2.2. *Isolation of psi-tectorigenin*

The actinomycetes strain SA1715 was cultured in a 500 ml Erlenmeyer flask containing 110 ml of medium consisting of 2% glucose, 2% soybean meal and 0.1% $CaCO_3$ (pH 7.6) on a rotary shaker at 27°C for 48 h; 3.0 ml of the cultured broth was then inoculated into a 500 ml flask containing 110 ml of the above medium. The fermentation was carried out at 27°C for 4 days. The broth filtrate was extracted with butyl acetate and the extract concentrated in vacuo. The dried material was dissolved in $CHCl_3$ and applied onto a silica gel column. After washing with $CHCl_3$, the active fraction was eluted with $CHCl_3$-MeOH (100:1). The eluate was dried and then dissolved in a small amount of methanol for separation by Toyopearl HW-40 column chromatography. The active component was further purified by reverse-phase high-performance liquid chromatography using 60% acetonitrile as an eluent.

2.3. *Phosphatidylinositol turnover assay*

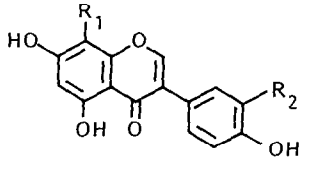
A431 cells (3×10^5) grown for 16 h beforehand were preincubated in 1 ml Hepes-buffered saline (HBS) (20 mM Hepes, 150 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 0.1% glycerol) containing [^{3}H]inositol (1 μCi/ml) at 37°C for 30 min. Then, a test chemical and EGF (400 ng/ml) were added, and the incubation was continued at 37°C for 60 min. Subsequently, 0.5 ml of 10% trichloroacetic acid containing 0.01 M sodium pyrophosphate was added, and the acid-insoluble fraction was scraped off from the dish in 1.0 ml H_2O. The lipid was extracted from it by the addition of $CHCl_3$ and CH_3OH (1:1) and [^{3}H]inositol-labeled lipids counted by liquid scintillation spectrophotometry.

$^{32}P_i$ incorporation into phospholipids was assayed as follows. A431 cells (5×10^5 cells) were plated in 35-mm dishes 16 h before use and washed with HBS. The cells were incubated in 1.0 ml HBS containing 20 μCi $^{32}P_i$ with psi-tectorigenin and/or EGF at 37°C for 30 min, after which 0.5 ml of 10% trichloroacetic acid was added. The cells were scraped off and the lipids extracted with $CHCl_3$ and MeOH. Lipid extracts labeled with $^{32}P_i$ were concentrated and spotted on a silica gel F thin-layer chromatography plate. The plate was developed with chloroform-methanol-acetic acid-H_2O (25:15:4:2). Radioactive spots were identified by autoradiography.

2.4. *Tyrosine protein kinase assay [11]*

The reaction mixture containing 1 mg/ml of a synthetic peptide (RR-SRC), 8.3 μCi/ml of [γ-^{32}P]ATP, 50 ng/ml of EGF and the membrane fraction of A431 cells with or without inhibitor was incubated at 0°C for 30 min in 60 μl of 10 mM Hepes buffer (pH 7.2). The reaction was then stopped by the addition of 25 μl of 10% trichloroacetic acid and 6 μl bovine serum albumin at 10 mg/ml. After incubation at 0°C for 30 min, the mixture was centrifuged (13000 rpm, 5 min). Thereafter, 45 μl of the supernatant was applied to a 1.5 × 1.5 cm piece of P81 phosphocellulose paper. The paper was then washed in 30% acetic acid for 15 min, 3 times in 15% acetic acid at room temperature, and finally rinsed in acetone. It was dried and counted for radioactivity.

In situ inhibitory activity of psi-tectorigenin against tyrosine kinase was examined as follows: A431 cells (5×10^5) were grown in 35-mm dishes for 18 h before use. The cells were washed twice with phosphate-free Dulbecco's modified Eagle medium (DMEM) supplemented with 4% dialyzed serum and then labeled for 4 h with 150 μCi $^{32}P_i$ in 1 ml phosphate-free medium. Then, psi-tectorigenin and/or EGF were added. After 30 min, cells were extracted with 0.5 ml Radio-immune precipitation (RIPA) buffer. The clarified cell extract (100 μl) was incubated for 2 h at 0°C with a saturating amount (10 μl) of monoclonal anti-EGF receptor antibody and then 200 μl of a suspension of *Staphylococcus aureus* Cowan I was added, and the mixture allowed to stand at 4°C overnight. The bacterial cells adsorbing the immune complexes were then washed with RIPA buffer 5 times at 4°C, suspended in 50 μl electrophoresis loading buffer, boiled for 5 min, and centrifuged at 15000 × *g* for 1 min. The supernatant was electrophoresed on an SDS-polyacrylamide gel, after which the gel was stained with Coomassie blue, dried, and autoradiographed.



	R_1	R_2
psi-tectorigenin	OMe	H
orobol	H	OH
genistein	H	H

Fig.1. Structure of isoflavonoids.

3. RESULTS

A culture filtrate from an actinomycetes strain found in a river near Shanghai showed strong in-

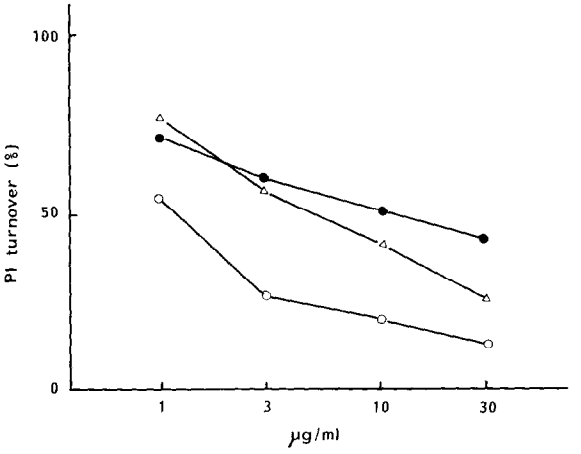


Fig.2. Inhibition of phosphatidylinositol turnover by psi-tectorigenin and other isoflavonoids. Psi-tectorigenin (○), orobol (●) or genistein (△) was added to cultured A431 cells preincubated with labeled inositol for 60 min with EGF. The lipid fraction was extracted and counted. Control value was 9245 cpm.

Table 1

Effect of psi-tectorigenin on EGF-induced phosphatidylinositol turnover

	$^{32}P_i$ incorporation[a] cpm(%)	
	PI	PA
None	224	1161
EGF	4704	6779
+ 5 μg/ml psi-tectorigenin	764	1666
+ 10	281	1651
+ 50	510	1984

[a] A431 cells were incubated with psi-tectorigenin and labeled sodium phosphate for 30 min

PI, phosphatidylinositol; PA, phosphatidic acid

hibition against PtdIns turnover. The active principle was purified and its structure was found to be identical with that of psi-tectorigenin by NMR (fig.1).

As shown in fig.2, psi-tectorigenin inhibited EGF-induced inositol incorporation with an IC_{50} of about 1 μg/ml in our assay system. The inhibitory activity of psi-tectorigenin was several times stronger than that of orobol or genistein.

Inhibition of PtdIns turnover by psi-tectorigenin was further confirmed by P_i incorporation into phospholipids. Incorporation of P_i into both phosphatidic acid and phosphatidylinositol was markedly enhanced by EGF in A431 cells, and addition of 5–50 μg/ml of psi-tectorigenin inhibited formation of phosphatidic acid by about 90% and that of PtdIns by 90–99%, as shown in table 1.

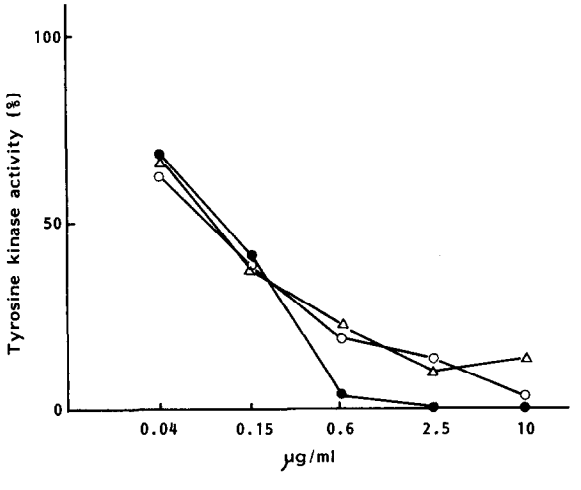


Fig.3. Inhibition of EGF receptor tyrosine kinase by isoflavonoids. The reaction mixture containing psi-tectorigenin (○), orobol (●) or genistein (△) with A431 cell membranes, peptide substrate and γ-labeled ATP was incubated for 30 min at 0°C. The labeled peptide was collected on phosphocellulose paper and its radioactivity counted. Control value was 2331 cpm.

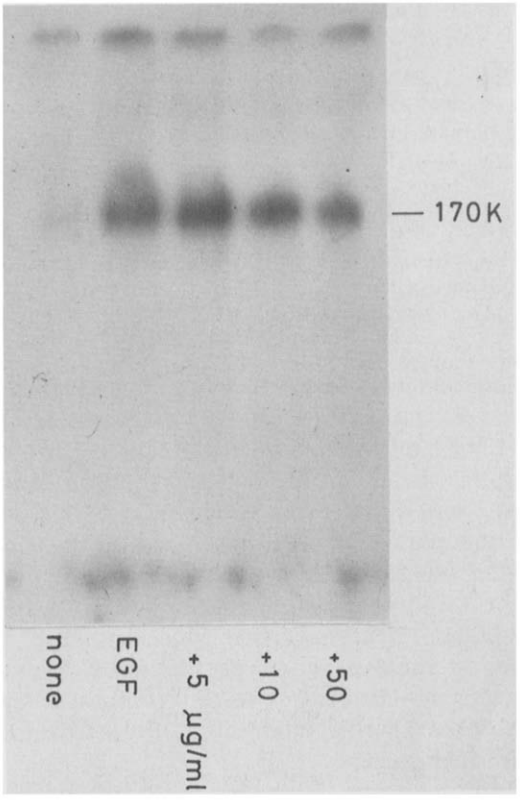


Fig.4. Effect of psi-tectorigenin on EGF receptor tyrosine kinase. Cultured A431 cells were supplemented with psi-tectorigenin for 30 min. The labeled EGF receptor (170 kDa) was precipitated and subjected to SDS gel electrophoresis.

Psi-tectorigenin, orobol, and genistein all showed similar in vitro inhibitory activity against EGF receptor tyrosine protein kinase, with an IC_{50} of about 0.1 μg/ml as shown in fig.3. However, psi-tectorigenin did not inhibit EGF receptor tyrosine kinase at 50 μg/ml in situ, as shown in fig.4.

4. DISCUSSION

Psi-tectorigenin was first described in 1953 by Baker et al. [12] as a synthetic isomer of tectorigenin. Later it was isolated from a culture

broth of *Aspergillus niger* NRRL-3122 as a weak inhibitor of dopa decarboxylase [13]. Recently, orobol [1] and genistein [14], which have a structure related to that of psi-tectorigenin, have been isolated from *Streptomyces* as inhibitors of tyrosine kinase. Genistein was also reported by Akiyama et al. [15] to inhibit EGF-induced PtdIns turnover. We found that orobol also inhibited PtdIns turnover with a similar IC_{50} value. However, the IC_{50} of psi-tectorigenin for PtdIns turnover was several times lower than those of genistein and orobol.

Genistein was reported to inhibit both PtdIns turnover and EGF receptor tyrosine kinase at 40–100 μg/ml in cultured A431 cells [15,16]. On the other hand, an immunoprecipitation experiment showed that psi-tectorigenin at 5–50 μg/ml did not markedly inhibit EGF receptor tyrosine protein kinase in cultured A431 cells, but only PtdIns turnover. In situ inhibition of tyrosine protein kinase requires a higher concentration of the inhibitor than in vitro possibly due to slow penetration. Therefore, psi-tectorigenin is a more potent and specific inhibitor of PtdIns turnover than is genistein.

Since no potent inhibitors of PtdIns turnover have been previously reported, psi-tectorigenin should prove to be a useful tool in functional studies of PtdIns turnover.

Acknowledgement: This work was partly supported by a Grant-in-Aid for New Drug Development Research from the Ministry of Health and Welfare, Japan.

REFERENCES

[1] Umezawa, H., Imoto, M., Sawa, T., Isshiki, K., Matsuda, N., Uchida, T., Iinuma, H., Hamada, M. and Takeuchi, T. (1986) J. Antibiot. 39, 170–173.
[2] Imoto, M., Umezawa, K., Sawa, T., Takeuchi, T. and Umezawa, H. (1987) Biochem. Int. 15, 989–995.
[3] Sayer, S.T. and Cohen, S. (1981) Biochemistry 20, 6280–6286.
[4] Habenicht, A.J.R., Glomset, J.A., King, W.C., Nist, C., Mitchell, C.D. and Ross, R. (1981) J. Biol. Chem. 256, 12329–12335.
[5] Farrar, W.L. and Anderson, W.B. (1985) Nature 315, 233–235.
[6] Nishizuka, Y. (1984) Nature 308, 693–698.
[7] Berridge, M.J. and Irvine, R.F. (1984) Nature 312, 315–321.
[8] Fleischman, L.T., Chahwala, S.B. and Cantley, L. (1986) Science 231, 407–410.
[9] Kaplan, D.R., Whitman, M., Schaffhausen, B., Raptis, L., Garcea, R.L., Pallas, D., Roberts, T.M. and Cantley, L. (1986) Proc. Natl. Acad. Sci. USA 83, 3624–3628.
[10] Jackowski, S., Rettenmier, C.W., Sherr, C.J. and Rock, C.O. (1986) J. Biol. Chem. 261, 4978–4985.
[11] Imoto, M., Umezawa, K., Isshiki, K., Kunimoto, S., Sawa, T., Takeuchi, T. and Umezawa, H. (1987) J. Antibiot. 40, 1471–1473.
[12] Baker, W., Dunstan, I., Harborhe, J.B., Ollis, W.D. and Winter, R. (1953) Chem. Ind. 1953, 277.
[13] Umezawa, H., Tobe, H., Shibamoto, N., Nakamura, F., Nakamura, K., Matsuzaki, M. and Takeuchi, T. (1975) J. Antibiot. 28, 947–952.
[14] Ogawara, H., Akiyama, T., Ishida, J., Watanabe, S. and Suzuki, K. (1986) J. Antibiot. 39, 606–608.
[15] Akiyama, T. and Ogawara, H. (1987) Seikagaku 59, 1016–1020.
[16] Akiyama, T., Ishida, J., Nakagawa, S., Ogawara, H., Watanabe, S., Itoh, N., Shibuya, M. and Fukami, Y. (1987) J. Biol. Chem. 262, 5592–5595.