INHIBITION OF EPIDERMAL GROWTH FACTOR-INDUCED ACTIVATION OF PHOSPHOLIPASE C BY PSI-TECTORIGENIN

Sir:

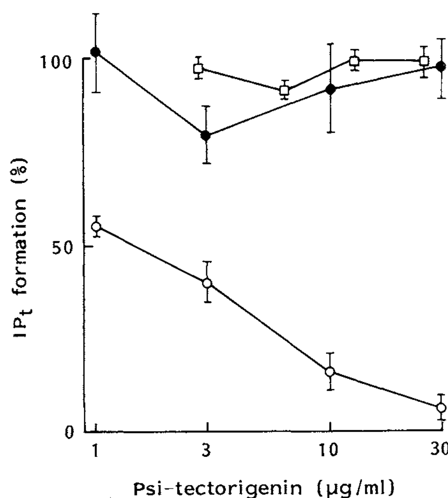
We have isolated psi-tectorigenin from *Nocardiopsis*, as an inhibitor of phosphatidylinositol turnover (PI turnover). It inhibited epidermal growth factor (EGF)-induced inositol incorporation into inositol lipids with an IC_{50} of about 1.0 μg/ml in human epidermoid carcinoma A431 cells[1]. We have studied the mechanism of action and found that psi-tectorigenin acts on the activation of phospholipase C, a rate limiting enzyme of PI turnover.

First, we analyzed the effect of psi-tectorigenin on agonist-induced inositol phosphates production. A431 cells (3×10^5) grown for 16 hours beforehand in 24-well plates were prelabeled with myo-[^{3}H]inositol (1 μCi/ml, 18.3 Ci/mmol, Amersham) for 24 hours in inositol-free DULBECCO's modified EAGLE's medium (DMEM) containing 10% dialyzed calf serum. NIH3T3 cells were grown for 2 days before addition of inositol. Then, the medium was removed and the cells were preincubated for 15 minutes in 0.5 ml of inositol-free DMEM containing 30 mM LiCl. Psi-tectorigenin was then added, and after 15 minutes EGF (400 ng/ml) or 20 μM of ATP was added to A431 cells, or bombesin (10 μM) was added to NIH3T3 cells, and each incubation was continued for a further 5 minutes. The reaction was terminated by the addition of ice-cold 10% $HClO_4$, and the mixture was then neutralized by addition of 1.53 M KOH in 75 mM HEPES. The solution was kept on ice for 15 minutes after which it was centrifuged at 10,000 rpm for 10 minutes at 4°C. The supernatant was applied to an Amprep SAX column (Amersham), and the column was then washed with water and eluted with 0.17 M $KHCO_3$. The obtained eluate which contains inositol phosphates ($IP+IP_2+IP_3$) was counted in a liquid scintillation counter. Psi-tectorigenin inhibited EGF-induced inositol phosphates production in a dose-dependent manner and the IC_{50} value was about 1.0 μg/ml, and complete inhibition was observed at 30 μg/ml (Fig. 1). However, psi-tectorigenin did not inhibit ATP-induced formation of inositol phosphates in A431 cells. It also showed no effect on bombesin-induced inositol phosphates formation in NIH3T3 cells (Fig. 1). Both ATP and bombesin are considered to act by a tyrosine kinase-independent mechanism[2,3].

Next we measured the effect of psi-tectorigenin on *in vitro* phospholipase C activity. The assay method was described before[4]. Crude homogenate after removal of nuclear fraction of A431 cells treated with EGF (400 ng/ml) for 10 minutes was used as a phospholipase C preparation. It was incubated with [^{3}H]phosphatidylinositol 4,5-diphosphate ([^{3}H]PIP_2) in the presence or absence of various concentrations of psi-tectorigenin at 37°C for 15 minutes. The reaction was terminated by the addition of 10% TCA and 1% bovine serum albumin. The precipitate was removed by centrifugation, and phospholipase C activity was evaluated by the radioactivity of the TCA soluble fraction containing [^{3}H]IP_3. Psi-tectorigenin did not affect the activity of phospholipase C up to 10 μg/ml. These results indicated that psi-tectorigenin inhibited phospholipase C indirectly.

Then we looked into the effect of psi-tectorigenin on the activation of phospholipase C. We found that the *in vitro* phospholipase C activity in homogenate

Fig. 1. Effects of agonist-induced inositol phosphate production by psi-tectorigenin in A431 cells or NIH3T3 cells.



After pretreatment of cells with verious concentrations of psi-tectorigenin for 15 minutes at 37°C, EGF (400 ng/ml; ○) or ATP (20 μM; ●) was added to A431 cells for 5 minutes. Bombesin (10 μM; □) was added to NIH3T3 cells. Total inositol phosphates were measured as described in text. The 100% and 0% values (dpm) were 1,408.7±16.4 and 504.4±101.5, 1,281.48±45.98 and 889.15±40.13, and 2,935.45±323.94 and 1,304.31±225.38, for EGF, ATP and bombesin, respectively. Values are means±SD of triplicate samples.

prepared from EGF-treated A431 cells was 4-fold higher than that from untreated cells, and that EGF failed to activate the enzyme in the presence of psi-tectorigenin (Table 1). Thus, psi-tectorigenin was shown to inhibit the activation step of phospholipase C.

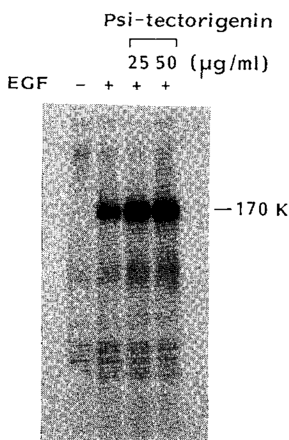
Table 1. Inhibition of EGF-induced phospholipase C (PLC) activation by psi-tectorigenin.

Addition	PLC activity (dpm)
None	332.8 ± 14.2
+ Psi-tectorigenin	547.2 ± 55.0
+ EGF	1338.0 ± 14.2
+ EGF + psi-tectorigenin	609.6 ± 11.5*

A431 cells were treated or not with psi-tectorigenin (25 μg/ml) in the presence of Na_3VO_4 (100 μM) for 15 minutes, and then EGF (400 ng/ml) was added. After 5 minutes, the cells were collected, homogenized and centrifuged at 500 × g for 10 minutes. The phospholipase C activity in the supernatant obtained was determined as described in text. Values are means ± SD of triplicate samples.

* $P < 0.02$.

Fig. 2. Effect of psi-tectorigenin on tyrosine phosphorylation of EGF receptor.



A431 cells (3 × 10^5) grown for 16 hours beforehand in 35 mm dish were prelabeled for 4 hours with 100 μCi of ^{32}Pi in phosphate-free DMEM containing 4% dialyzed serum. Psi-tectorigenin was added to cells and preincubated for 15 minutes, then EGF (100 ng/ml) was added. After 5 minutes, the cells were solubilized with 0.5 ml of cold RIPA buffer[6]. Aliquot of cell lysate was incubated with Sepharose-linked anti-phosphotyrosine monoclonal antibody (Oncogene Science, Inc.) at 4°C overnight. Immune complex obtained was electrophoresed on SDS-PAGE. The tyrosine phosphorylated proteins were detected by autoradiography.

Activation of phospholipase C by EGF is considered to involve EGF receptor tyrosine kinase activation[3,5]. In fact, erbstatin, a tyrosine kinase inhibitor, inhibits EGF-induced phospholipase C activation[4]. However, psi-tectorigenin does not inhibit EGF-induced receptor autophosphorylation as detected by EGF receptor antibody[1] and we have confirmed it by using anti-phosphotyrosine antibody (Fig. 2). Psi-tectorigenin at 25 and 50 μg/ml rather increased tyrosine phosphorylation of the receptor slightly. Therefore, psi-tectorigenin should inhibit another process for activation of phospholipase C rather than tyrosine kinase. The mechanism of phospholipase C activation may include a more complex pathway. Psi-tectorigenin may be a suitable tool to elucidate this complex mechanism.

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