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# Tyrphostins inhibit the epidermal growth factor receptor-mediated breakdown of phosphoinositides

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In response to epidermal growth factor (EGF) and the $Ca^{2+}$ ionophore A23187, the total phosphatidylinositides ($IP_T$) increased in A431 human epidermoid carcinoma cells 1.8- and 2.0-fold and in the EGF-dependent A431/Clone 15-2 cells 3.0- and 8.0-fold, respectively, over basal levels. Both responses were inhibited by the antiproliferative agents tyrphostins, but the EGF-induced increase in $IP_T$ was inhibited to a much greater extent than that induced by the ionophore. Tyrphostins which are potent EGF-receptor kinase inhibitors were also potent in blocking the EGF-induced production of phosphoinositides. The less potent tyrphostins were found to inhibit the EGF-dependent $IP_T$ formation more weakly. These results support the notion that phospholipase C is activated through its phosphorylation by the EGF receptor.

Tyrphostins; Epidermal growth factor; Phospholipase C phosphorylation; $Ca^{2+}$ ionophore; (A431 cell, A431/Clone 15–2 cell)

## 1. INTRODUCTION

Signal transduction in response to many hormones and growth factors in a variety of mitogenically responsive cell lines leads to enhanced phospholipase C (PLC)-catalyzed hydrolysis of phosphatidylinositol 4,5-diphosphate ($PIP_2$) to diacylglycerol (DAG) and inositol phosphates [1]. Inositol (1,4,5-triphosphate) and DAG act as second messengers: the former causes the release of intracellular $Ca^{2+}$ and the latter activates protein kinase C [1,2]. The increased cytosolic inositol phosphates, DAG and $Ca^{2+}$ in response to receptor activation by the mitogen epidermal growth factor (EGF) [1–6] was significant in the human epidermoid carcinoma cell line A431 [7,8], whose membranes contain 20–50-fold the normal concentration of EGF receptors (EGFR) [9]. The proliferation of these cells is blocked by EGF, while the growth of the cell line A431/Clone 15 is EGF-dependent [10]. The activity of PLC immunopurified from extracts of EGF-stimulated A431 cells on Sepharose-linked antiphosphotyrosine increased ten-fold over controls, suggesting a direct biochemical link between the EGF-receptor kinase activity and $PIP_2$ turnover and that the EGF receptor directly phosphorylates PLC on tyrosine residues leading to its activation [11]. The link between the EGFR and the phosphoinositide pathway was also suggested from studies on the kinase deficient mutant of the receptor. A point mutation at the ATP-binding site which nullifies the PTK activity of the receptor also abolishes the EGF-induced formation of inositol phosphates [12]. Indeed, Margolis et al. [13] described the phosphorylation of PLC-II in response to EGF and proposed that this event might enhance the enzymatic activity of PLC and concluded that the EGF-induced tyrosine phosphorylation of PLC-II might be an important regulatory link between the kinase activity of the EGF receptor and the hydrolysis of $PIP_2$. Meisenhelder et al. [14] have described the phosphorylation of PLC-II (PLC $\gamma$) in response to PDGF and EGF and also proposed that phosphorylation of PLC-II (PLC $\gamma$) by PDGF and EGF receptors leads to its activation and to a consequent increase in $PIP_2$ turnover. Activation of PLC by EGFR and PDGFR may be responsible, at least partially, for the mitogenic signal generated by these growth factors. In support of this hypothesis is the recent report that microinjection of PLC-II into NIH 3T3 cells induces a growth and oncogenic potential in growth arrested fibroblast cells [15].

Selective anti-proliferative compounds referred to as tyrphostins have been developed in our laboratory [16,17]. One group, of the benzenemalononitrile family, is specific for the EGF-receptor kinase [17] and, therefore, inhibits EGF-dependent cell proliferation [16,18]. These compounds were also shown to inhibit

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*Abbreviations:* PLC, phospholipase C; $PIP_2$, phosphatidylinositol 4,5-diphosphate; $IP_T$, total phosphatidylinositides; EGF, epidermal growth factor; EGFR, EGF receptor; PDGF, platelet derived growth factor; EBSS, Earle's basal salt solution; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum

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the phosphorylation of intracellular target proteins [18]. Especially interesting was the demonstration that a tyrphostin such as RG50864 (AG213) inhibits the EGF-dependent phosphorylation of PLC-II (PLC $\gamma$) in cells which harbor about $10^6$ receptors/cell [13]. In this communication we demonstrate that tyrphostins which block EGF-dependent kinase activity also block EGF-dependent breakdown of $PIP_2$ strengthening the hypothesis that PLC-II phosphorylation by EGFR results in its activation.

## 2. MATERIALS AND METHODS

### 2.1. *Materials*

EGF, $Ca^{2+}$ ionophore A23187, vitamins, amino acids and Earle's basal salt solution (EBSS) were purchased from Sigma Chemical Co.; *myo*-[2-$^3$H]inositol was purchased from ARC, Inc., St. Louis, MO; Dowex AG 1×8 (200–400 mesh, formate form) was obtained from Bio-Rad Laboratories; Dulbecco's modified Eagle's medium (DMEM) and fetal calf serum (FCS) were from Gibco Ltd, Paisley, Scotland. A431/Clone 15-2 (A431/C115-2) cells were a sub-clone of the A431/Clone 15 cells [10] which were a kind gift from Dr R. Bravo (EMBL, Heidelberg).

### 2.2. *Methods*

A431 and A431/C115-2 cells were grown in 82 cm$^2$ flasks in DMEM supplemented with penicillin (100 U/ml) and streptomycin (100 $\mu$g/ml) and containing 5% or 10% inactivated (FCS) plus 100 ng/ml EGF, respectively. When confluent, cells were plated on 12×22 mm diameter dishes in 1.6 ml DMEM per well: $8\times10^4$ A431 cells were plated in media containing 5% FCS and $2\times10^4$ A431/C115-2 cells plated in media containing 10% fetal-calf serum and 20 ng/ml EGF. Two days later, when cells reached near confluence, the cells were washed twice with PBS and 0.8 ml inositol-free EBSS supplemented with vitamins and amino acids as in DMEM and containing 0.5–2 $\mu$Ci [$^3$H]inositol/ml plus the desired concentration of tyrphostin were added to each well. Tyrphostins were dissolved in DMSO and then diluted with EBSS to a final DMSO concentration of 1.25 $\mu$l/ml. After 24 h, the media were washed twice with PBS and 0.8 ml fresh EBSS containing 100 ng/ml EGF, the desired concentration of tyrphostin and 10 mM LiCl were added to each well. After 20–40 min at 37°C, 0.8 ml cold 10% TCA was added to terminate the reaction and total phosphatidylinositides ($IP_T$) were determined according to Berridge et al. [19]. Data represent averages of 2–5 experiments performed in duplicate. Data were calculated and plotted with the aid of the EZ-FIT program (courtesy of Frank W. Perrella of E.I. DuPont de Nemours & Co.).

## 3. RESULTS

$IP_T$ formation in response to EGF was determined at different time intervals both in A431 and A431/C115-2 cells and was found to increase linearly up to 20 and 40 min, respectively. In the A431/Clone 15-2 cells, $IP_T$ continued to rise for at least 10 h after EGF stimulation. [$^3$H]$PIP_2$ breakdown in response to EGF increased with EGF concentrations and was maximal at 100 ng/ml (not shown) and, therefore, this dose of the growth factor was used in all subsequent experiments.

The effect of tyrphostin AG18 (3,4-dihydroxybenzene malononitrile) on the response of PLC to EGF in A431 and A431/C115-2 cells is compared in fig.1. In

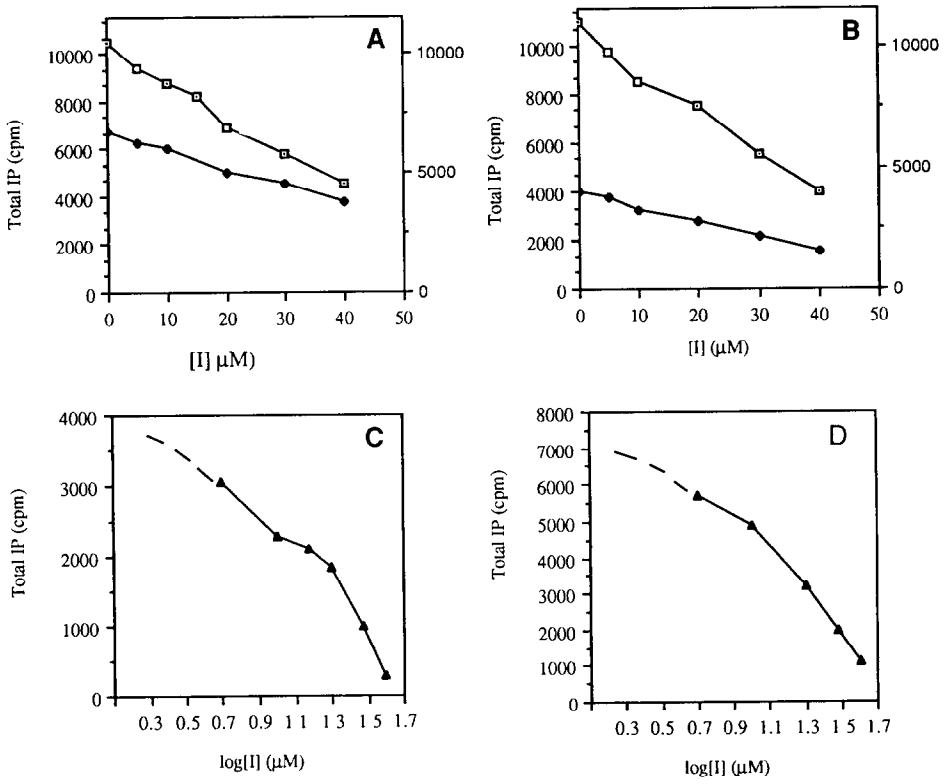



Fig.1. The inhibition of $PIP_2$ hydrolysis by tyrphostin AG18. The accumulation of inositol phosphates in the absence (♦) and in the presence (⊡) of EGF and the net increase in response to EGF (▲) in A431 cells (A and C) or A431/C115-2 (B and D).

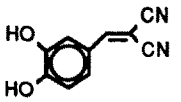
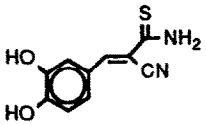
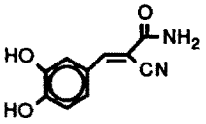
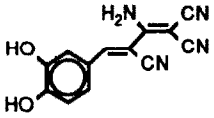
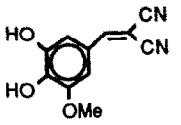
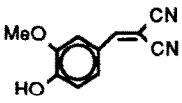
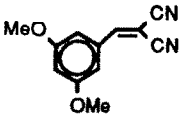
response to EGF, $IP_T$ increased 1.5–1.8-fold in A431 cells and 3.0-fold or more in the A431/C115-2 cells. In both cell types, the tyrphostin inhibited 'basal' [$^3$H]$IP_T$ formation as well as the EGF-stimulated activity (fig.1). The $IC_{50}$ for the inhibitory effect on the EGF-dependent [$^3$H]$IP_T$ formation was found to be 14.5 $\mu$M for the parent cells (A431) and 18.2 $\mu$M for the A431/C115-2 cells. Similar behavior was found for a number of other tyrphostins (table 1). Fig.2 (A and B) shows the effect of tyrphostin AG18 on the [$^3$H]$IP_T$ formation induced by $Ca^{2+}$ ionophore A23187 in A431 and A431/C115-2 cells. In both cell types, the effect of the tyrphostin was far greater on the EGF-dependent than on the $Ca^{2+}$ ionophore-induced breakdown of $PIP_2$. The discrepancy between the inhibitory effect of tyrphostins on the EGF-dependent activity and $Ca^{2+}$ ionophore-dependent activity was highest for the better inhibitors: AG18, AG213, AG99 and AG114; poor EGF receptor kinase inhibitors (AG115 and AG81) showed diminished discrimination (table 1).

## 4. DISCUSSION

Recent studies have shown that in response to EGF and PDGF, PLC-II becomes phosphorylated on tyrosine residues [12,13]. In these studies the authors suggest that phosphorylation of PLC-II (PLC $\gamma$) in response to growth factor-receptor activation results in

Table 1

Inhibition of EGF dependent $PIP_2$ hydrolysis by tyrphostins

| Tyrphostin | | Inhibition of $PIP_2$ hydrolysis, $IC_{50}$ ($\mu$M) | | Inhibition of EGFR-TK activity, $IC_{50}$ ($\mu$M) | |
|---|---|---|---|---|---|
| Structure | No. | EGF | $Ca^{2+}$ ionophore | Poly-Gat[a] | Autophosp-horylation[b] |
|  | AG18 | 18.4 | ⩾85.0 | 10.0 | 40.0 |
|  | AG213 | 19.6 | ⩾90.0 | 1.8 | - |
|  | AG99 | 38.0 | ⩾100.0 | 12.5 | 4.0 |
|  | AG114 | 55.0 | ⩾100.0 | 2.5 | 18 |
|  | AG34 | 25.0 | 58.0 | 6.0 | 20 |
|  | AG115 | 66.0 | ⩾85 | 200.0 | - |
|  | AG81 | 85.0 | ⩾110.0 | 1250.0 | - |

[a] Concentration needed to inhibit 50% of Poly($Glu_6Ala_3Tyr$) phosphorylation at substrate concentration which equals $2K_m$, as described in [16]

[b] Inhibition of EGF receptor autophosphorylation, as described in [16,17]

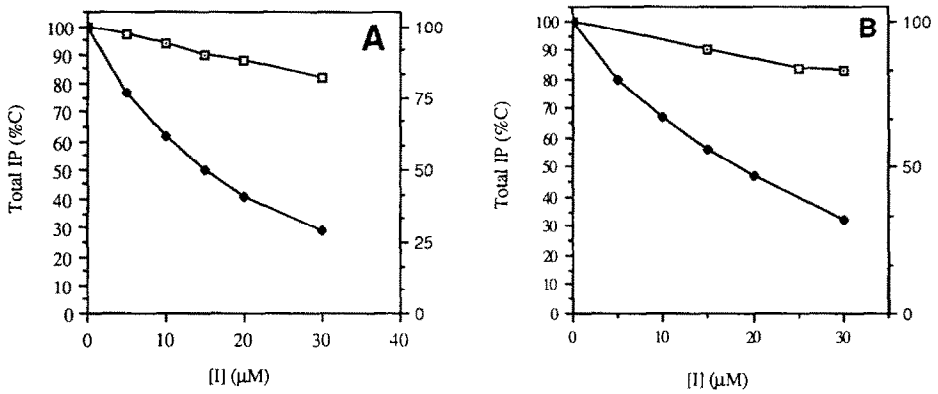



Fig.2. The inhibition of $PIP_2$ hydrolysis by tyrphostin AG18 in (A) A431 and (B) A31/Clone 15-2 cells in response to EGF (♦) or $Ca^{2+}$ ionophore A23187 (□). %C = percent control.

enzyme activation leading to the well documented growth factor-induced breakdown of $PIP_2$ and $Ca^{2+}$ mobilization [1-6,13]. In one of these studies [13], it was shown that tyrphostin AG213 (table 1) inhibits the EGF-induced phosphorylation of PLC-II and the EGF-induced $Ca^{2+}$ mobilization. In the current report we demonstrate that tyrphostins which are effective EGFR-kinase inhibitors are also effective in the inhibition of EGF-dependent breakdown of $PIP_2$. Furthermore, the most potent tyrphostin inhibitors also inhibit the EGF-dependent $IP_T$ formation more potently than the EGF-independent activation of phospholipase C induced by the $Ca^{2+}$ ionophore A23187. We were surprised to find that tyrphostins actually inhibit basal $PIP_2$ breakdown. One possible explanation is that in cells which harbor an extremely high number of EGF receptors most of the PLC is physically associated with the receptor. Therefore, binding of tyrphostins to the EGF-receptor kinase substrate domain might affect the non-phosphorylated receptor-associated state of the enzyme. Indeed, it was shown in a previous study that the phosphorylation-dependent association between the EGFR and PLC-II was blocked by AG213 [13]. Thus, one may speculate that the basal activity of PLC within the EGFR-PLC-II complex is higher than the free form of PLC-II. It follows that at increasing tyrphostin concentrations basal PLC activity is progressively inhibited concomitantly with the breakdown of the PLC-EGFR complex. It is likely that in cells which harbor a smaller number of EGF receptors, PLC is not associated with the growth factor receptor and, therefore, its activity should not be influenced by tyrphostins. In fact, in PC12 cells which harbor only $8.4 \times 10^4$ EGF receptors/cell and respond weakly to EGF [20-22], we could not detect EGF-stimulated $PIP_2$ breakdown nor could we detect any effect of tyrphostins on PLC activity (unpublished).

The efficacy of AG213 is much lower in inhibiting EFG-induced breakdown of $PIP_2$ than in inhibiting the phosphorylation of polyGAT (table 1). The $IC_{50}$ for the inhibition of $PIP_2$ breakdown is in the same range as the $IC_{50}$ for the inhibition of the autophosphorylation of EGFR by the tyrphostin. This is not surprising, since the receptor and PLC-II (PLC $\gamma$) are complexed with each other. The situation with AG81 seems to be reversed: the tyrphostin is a more effective inhibitor of $PIP_2$ hydrolysis in intact cells, although it is a poor inhibitor of phosphorylation of an exogenous substrate in vitro. This reverse discrepancy most probably results from the accumulation of the very hydrophobic AG81 in the cells much beyond the initial concentration of the compound in the medium. A similar situation was encountered with insulin receptor kinase where hydrophobic inhibitors were found to be more effective in intact cells than in vitro [23]. In this case the hydrophobic compounds similarly accumulated inside the cells to concentrations beyond those of the medium.

In conclusion the observation that tyrphostins inhibit EGF-dependent $PIP_2$ hydrolysis strengthens the hypothesis that the phosphorylation of PLC-II by EGFR leads to its activation [13,14]. These studies as well as our previous reports [13,17,18,23] demonstrate the usefulness of tyrphostins as molecular tools for the dissection of the signals triggered by protein tyrosin kinases.

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