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Epidermal growth factor-induced increases in inositol trisphosphates, inositol tetrakisphosphates, and cytosolic Ca^{2+} in a human hepatocellular carcinoma-derived cell line

Ann Gilligan, Marc Prentki[+]*, Major C. Glennon* and Barbara B. Knowles

Wistar Institute of Anatomy and Biology, Philadelphia, PA 19104 and **Department of Biochemistry and Biophysics and Diabetes Research Center, University of Pennsylvania, Philadelphia, PA 19104, USA*

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A human hepatocellular carcinoma-derived cell line, PLC/PRF/5, was examined for its ability to respond to epidermal growth factor (EGF) exposure with increased phosphatidylinositol 4,5-bisphosphate hydrolysis. Upon addition of EGF (25 ng/ml), a rapid (10–15 s) but transient increase in $Ins(1,4,5)P_3$ levels and large, prolonged (2 min) increases in $Ins(1,3,4,5)P_4$ and $Ins(1,3,4)P_3$ levels were detected. Increases in cytosolic Ca^{2+} were observed after a 10 to 20 s lag, reaching peak value at 1 min, and remaining elevated for 10 min. The initial burst of cytosolic Ca^{2+} occurred in the absence of extracellular Ca^{2+} and probably reflects mobilization of intracellular Ca^{2+} stores. In cells pretreated with EGTA, the sustained component of the Ca^{2+} response was not observed. Comparison of the inositol phosphate and Ca^{2+} responses of PLC/PRF/5 cells to responses reported in other cell types indicates that this cell line is a good model for EGF action in liver.

Epidermal growth factor; Inositol phosphate metabolism; Cytosolic Ca^{2+}; Phosphatidylinositol 4,5-bisphosphate hydrolysis; (Human hepatoma cell; PLC/PRF/5)

1. INTRODUCTION

Epidermal growth factor (EGF) acts as a mitogen for several cell types; however, the relationship between EGF receptor-ligand binding and induction of cell proliferation remains undefined [1,2]. The EGF receptor possesses an intrinsic tyrosine-specific protein kinase which is stimulated upon ligand binding and results in receptor autophosphorylation and phosphorylation of several specific cellular substrates [3–6]. Mutations that abolish the receptor kinase activity block EGF induction of DNA synthesis in NIH 3T3 cells [7].

Correspondence address: A. Gilligan, Wistar Institute of Anatomy and Biology, Rm G54, 36th St at Spruce, Philadelphia, PA 19104, USA

[+] *Present address:* Institut de Biochimie Clinique, Centre Médical Universitaire, University of Geneva, Switzerland

Recent studies link EGF receptor activation to enhanced breakdown of phosphatidylinositols, a signalling pathway activated by a number of growth-promoting stimuli [8–14]. Diacylglycerol and inositol 1,4,5-trisphosphate ($Ins(1,4,5)P_3$) are formed upon hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) by phospholipase C and have been implicated in cellular regulation by their respective abilities to activate protein kinase C and mobilize intracellular stores of Ca^{2+} [15–18]. The metabolite of $Ins(1,4,5)P_3$, inositol 1,3,4,5-tetrakisphosphate ($Ins(1,3,4,5)P_4$), may also influence cytosolic Ca^{2+} levels [18].

The tyrosine-protein kinase and phospholipid signalling pathways may interconnect. Phosphatidylinositol (PI) kinase co-purifies with the EGF receptor [19] and the platelet-derived growth factor receptor, another tyrosine-specific protein kinase, has been shown to phosphorylate a putative PI kinase [20]. EGF appears to directly stimulate PI kinase activity in A431 cells [13] and

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to stimulate phospholipase C activity in A431 cells and rat hepatocytes [10–14]. However, this connection is still controversial and may depend upon cell type or the metabolic conditions. In one study, for instance, the EGF-induced rises in cytoplasmic Ca^{2+} observed in A431 cells were solely attributable to Ca^{2+} influx [21]. EGF also did not cause measurable increases in membrane polyphosphoinositides in rat liver preparations [22] or in inositol phosphates of BALB/C 3T3 cells [23].

In this report we described EGF-induced cytosolic Ca^{2+} fluxes and Ins(1,4,5)P_3 production in a differentiated human cell line, PCL/PRF/5. This hepatoma-derived cell line, which contains approximately 300000 EGF receptor molecules per cell, exhibits some of the properties of differentiated hepatocytes [24–26] and may provide a good model system for study of coupling between EGF receptor activation and PIP_2 hydrolysis.

2. MATERIALS AND METHODS

2.1. *Cell culture*

PLC/PRF/5 cells were cultured in Eagle's minimum essential medium (MEM) supplemented with 10% heat-inactivated fetal bovine serum and 2 mM glutamine. Cells were maintained and labelled in a humidified CO_2 incubator at 37°C.

2.2. *Inositol phosphate labelling*

PLC/PRF/5 cells were seeded at 1–1.5 × 10^6 cells per 100 mm dish. After 5 days, the medium was replaced with 10 ml of medium containing 3 μCi of *myo*-[2-^{3}H]inositol (Amersham, 20 Ci/mmol) per ml. After 24 h, this medium was exchanged for serum-free MEM containing 3 μCi of *myo*-[2-^{3}H]inositol per ml and incubated for another 18–22 h. Cells were washed twice with Dulbecco's phosphate buffered saline (PBS, Gibco), preincubated with serum-free MEM containing 0.1% bovine serum albumin (BSA) for 45 min at 37°C, and washed 3 times with PBS. Cells were then incubated with 25 ng/ml EGF (Collaborative Research, receptor grade) for 0, 10 to 15 s, or 2 min at 37°C and scraped after addition of 0.8 ml of ice-cold 10% trichloroacetic acid (TCA). The plates were washed once with 0.8 ml of 10% TCA and insoluble material removed by centrifugation. The TCA extracts were washed 4 times with water-saturated diethylether, lyophilized, stored at −70°C, and reconstituted with water prior to separation by high-pressure liquid chromatography (HPLC).

2.3. *HPLC analysis*

Inositol phosphates were separated on a Whatman Partisil Sax 10 column using a variation of the procedure of Irvine [27]. The following ammonium formate (pH 3.7 with phosphoric acid) elution sequence was used at a flow rate of 1.2 ml/min: 0.625 M for 9 min; 1.425 M from 10 to 18 min; 2.5 M from 20

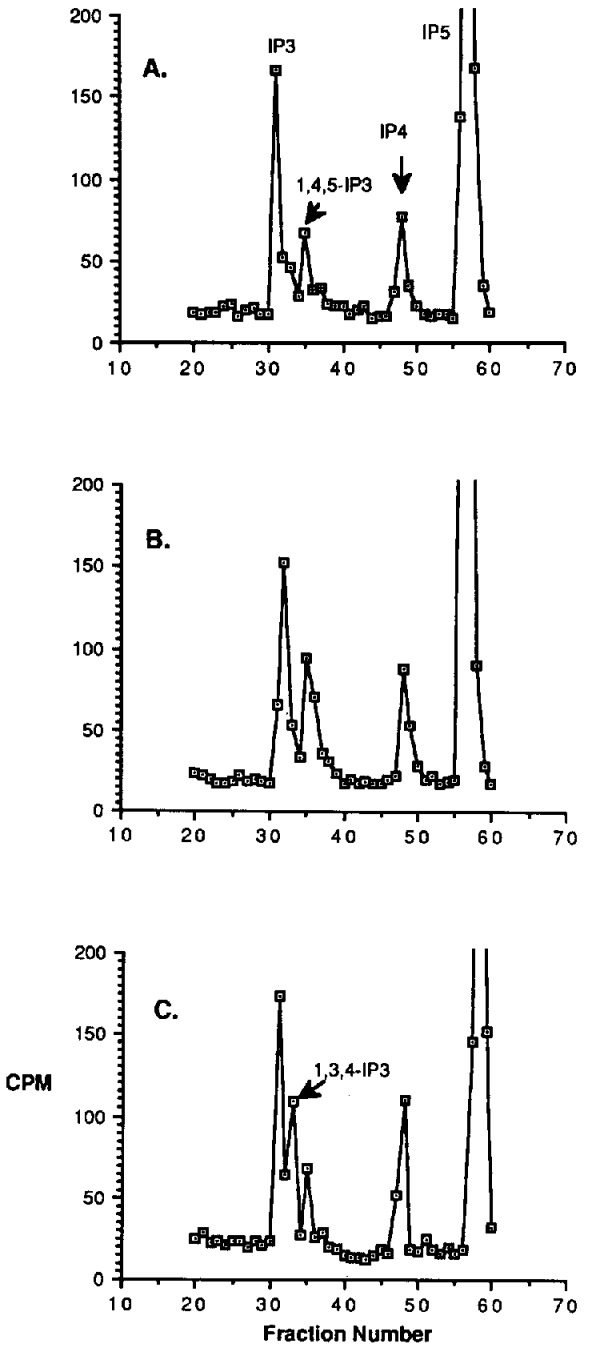

Fig.1. Separation of highly phosphorylated inositol phosphate isomers from EGF-treated cells by anion exchange HPLC. PLC/PRF/5 cells previously labelled with tritiated *myo*-inositol were treated with 25 ng/ml EGF for 0 (A), 10–15 s (B), and 2 min (C) and acid extracts of the cells injected into a Partisil Sax 10 HPLC column. 0.6 ml fractions were collected and tritium counts in each fraction measured.

to 35 min. Fractions (0.6 ml) were collected every 0.5 min. Fractions 1–39 were directly diluted with 0.9 ml of 50% methanol and mixed with 4.5 ml of ACS II scintillation fluid (Amersham). To avoid quenching of radioactivity due to high salt, 0.3 ml of each fraction beyond fraction 39 was replaced with 0.3 ml of water prior to the addition of 0.9 ml of 50% methanol. Samples were counted on the tritium channel of a Beckman scintillation counter.

2.4. Ca^{2+} measurements

The medium in a T-75 flask of PLC/PRF/5 cells was exchanged for serum-free MEM 1–2 days prior to confluence. 16 h later, cells were detached by incubation with 0.25% trypsin and 0.1% EDTA in PBS for 1–2 min at 37°C, diluted immediately with serum-free medium containing 0.25% BSA and 10 mM NaHepes (pH 7.4) (incubation medium), and centrifuged. Cells detached using EDTA alone gave similar results. After washing once, the cells were resuspended in 5 ml of the incubation medium and incubated at 37°C with 2 μM Fura 2/AM (Calbiochem-Behring) for 30 min. Cells were diluted, centrifuged, washed once, resuspended in 5 ml of incubation medium and incubated at 37°C for 15 min to complete intracellular conversion of Fura 2/AM to Fura 2. Cells were pelleted, resuspended in 200–400 μl of incubation medium and Ca^{2+} measurements made as previously described [28]. Briefly, the fluorescence of Fura 2-loaded cells was monitored using an MB-2 four filter air turbine fluorescence spectrometer (Johnson Foundation, University of Pennsylvania) at the excitation wavelengths of 340 nm (Ca^{2+}-Fura 2 complex) and 385 nm (free Fura 2) and at the emission wavelength of 510 nm. Because the fluorescence recordings at 385 nm had a better signal-to-noise ratio, results are presented using the free Fura 2 signal.

3. RESULTS

The ability of EGF to induce formation of Ins(1,4,5)P_3 in PLC/PRF/5 cells was investigated using HPLC chromatography to separate highly phosphorylated isomers of inositol. The chromatograms of acid-soluble extracts from cells treated with 25 ng/ml EGF for 0, 10–15 s, and 2 min are shown in fig.1. The data are uncorrected except for a dilution correction for the fractions 40 and above. Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4 peaks were identified by coinjection of known standards (New England Nuclear) and were present at significant levels in unstimulated cells. Substantial levels of 2 unidentified peaks were also observed. The first of these peaks migrated in the region where inositol trisphosphate elute, while the second eluted in high salt, after Ins(1,3,4,5)P_4. These peaks were tentatively identified as an inositol trisphosphate isomer ($InsP_3$) and inositol pentakisphosphate ($InsP_5$). 10–15 s after EGF addition, the same four peaks were prominent; however, the Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4 peaks appeared larger. At 2 min after addition of EGF, Ins(1,4,5)P_3 had decreased, Ins(1,3,4,5)P_4 remained elevated, and an inositol 1,3,4-trisphosphate (Ins(1,3,4)P_3) peak, identified by its chromatographic behavior and its characteristic delayed appearance upon stimulation [29–32], had become apparent.

Table 1 summarizes the results from three separate experiments. The inositol phosphate levels vary widely between experiments; however, all experiments show the same pattern of inositol phosphate metabolism. A consistent, though transient increase in Ins(1,4,5)P_3 was observed after EGF addition. Levels were between 2 and 3 times above basal 10–15 s after EGF addition but had returned to prestimulatory values after 2 min.

Table 1

Levels of highly phosphorylated inositol phosphate isomers in PLC/PRF/5 cells stimulated with epidermal growth factor

	Expt 1			Expt 2			Expt 3		
	0	10–15 s	2 min	0	10–15 s	2 min	0	10–15 s	2 min
$InsP_3$	101	109	100	215	176	205	264	264	259
Ins(1,3,4)P_3	0	0	51[a]	0	0	128[a]	0	0	79[a]
Ins(1,4,5)P_3	33	71[a]	32	36	102[a]	38	19	48[a]	19
$InsP_4$	52	58	67[a]	35	85[a]	148[a]	14	24[a]	70[a]
$InsP_5$	533	549	486	654	599	567	275	335	344

[a] Levels that differ from the 0 control (no EGF) by more than 25%

Cells were exposed to 25 ng/ml EGF for 0, 10 to 15 s, and 2 min. Values were corrected for background and dilution (fractions 40 and above) and expressed as cpm per mg of cell protein

Ins(1,3,4,5)P_4 increases were observed in some experiments 10–15 s after EGF addition, but substantial increases were always present after 2 min of stimulation. The production of Ins(1,3,4)P_3 was delayed, but this isomer was present at high levels 2 min after EGF addition.

The unassigned $InsP_3$ and $InsP_5$ peaks did not change by more than 25% upon EGF addition and their function is unknown. The very high levels of the unassigned $InsP_3$ and $InsP_5$ present in PLC/PRF/5 cells and the high levels of $InsP_5$ and $InsP_6$ present in other cell types upon long term labelling [32,33] make separation of the inositol phosphates into individual isomers essential. Protocols that do not enable separation of all isomers can lead to several underestimation of ligand-induced inositol phosphate increases [33]. In experiment no.2 of table 1, for example, the number of counts in the Ins(1,4,5)P_3 increased almost 3-fold, from 36 to 102, when cells were exposed to EGF for 10–15 s. At the same time, the counts in the three $InsP_3$ peaks combined increased by only 11%, going from 251 to 278.

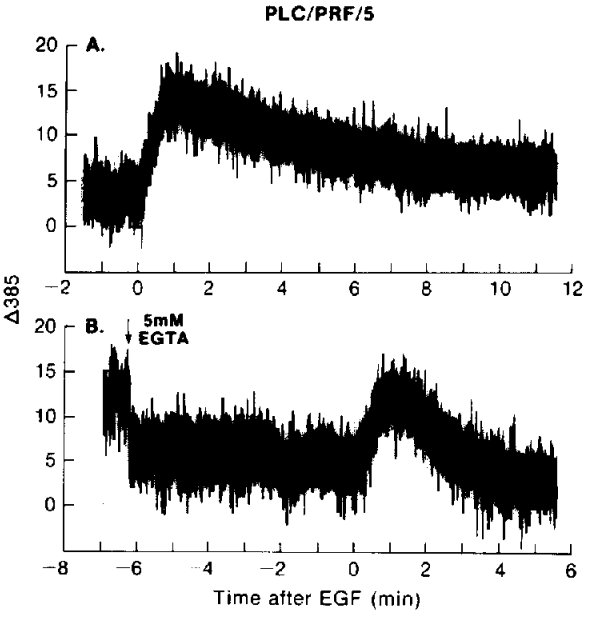

Fig.2. EGF-induced increases in cytosolic Ca^{2+} in the presence (A) and absence (B) of extracellular Ca^{2+}. Changes in fluorescence (arbitrary units) are plotted versus time after EGF (25 ng/ml) addition to PLC/PRF/5 cells. Fluorescence measurements shown are at excitation wavelength 385 nm and emission wavelength 510 nm. An increase in fluorescence indicates an increase in cytosolic Ca^{2+}. The instantaneous decrease in fluorescence observed upon EGTA addition occurs because of removal of Ca^{2+} from extracellular Ca^{2+}-Fura 2 complexes.

The physiological consequences of enhanced inositol phosphate production were assessed by measuring the EGF-induced changes in cytosolic Ca^{2+} in the absence and presence of 5 mM EGTA (fig.2). After a 10–20 s lag, EGF addition caused the cytosolic Ca^{2+} to increase from 114 nM to 306 nM (n = 6) within approximately 1 min (fig.2A). After an initial decrease from peak values, the cytosolic Ca^{2+} remained elevated for at least 10 min. Removal of extracellular Ca^{2+} by prior addition of EGTA only lowered peak Ca^{2+} values by 26% (n = 4); however, cytosolic Ca^{2+} returned to basal levels within 3 min (fig.2B).

4. DISCUSSION

The EGF-induced alterations in inositol phosphate levels in PLC/PRF/5 cells are similar to those observed in A431 cells exposed to EGF [10,14]. These patterns are consistent with initial stimulation of Ins(1,4,5)P_3 formation, phosphorylation of that isomer to form Ins(1,3,4,5)P_4, and formation of Ins(1,3,4)P_3 by an inositol 5′-phosphatase [29–32]. The transient nature of the Ins(1,4,5)P_3 increase may reflect Ca^{2+} stimulation of the kinase which converts it to Ins(1,3,4,5)P_4 [34,35] and protein kinase C activation of the 5′-phosphatase which converts it to inositol 1,4-bisphosphate [36]. Ins(1,3,4)P_3 production may be enhanced by EGF through stimulation of a similar 5′-phosphatase activity, as well as by the increased production of its precursor, Ins(1,3,4,5)P_4. The production of diacylglycerol which should result in protein kinase C activation is inferred from the high production of Ins(1,4,5)P_3 metabolites, indicating continual hydrolysis of PIP_2. Production of Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4 directly from $InsP_5$ has not been previously reported and is unlikely to have occurred here, since $InsP_5$ levels did not change by more than 25% within 2 min of EGF addition.

The change in Ca^{2+} levels in the EGF-treated cells indicates that the changes in inositol phosphates are physiologically significant. The initial rise in cytosolic Ca^{2+} occurs after the peak in Ins(1,4,5)P_3 and is probably due to mobilization of endoplasmic reticulum Ca^{2+} stores by this $InsP_3$

isomer. The later component of the Ca^{2+} response depends upon the presence of extracellular Ca^{2+} and may be contributed by influx across Ins(1,3,4,5)P_4-activated plasma membrane Ca^{2+} channels [30,37,38]. The dual component of the Ca^{2+} response to EGF in this cell line is similar to that observed in the A431 cell line and in primary rat hepatocytes [10–12] and may be a uniform response to EGF in cells where EGF receptor coupling to PI hydrolysis is intact.

Our results and those obtained recently in primary rat hepatocytes [11] argue against a requirement for very high levels of EGF receptor in the EGF-stimulated production of inositol phosphates. The response of PLC/PRF/5 cells to EGF was similar to the primary rat cell response; however, the primary rat hepatocytes showed sustained increases in Ins(1,4,5)P_3 production [11] whereas our cells did not. This difference may reflect the use, in that study, of EGF at doses 15-fold higher than those in our study and also the preincubation of the primary rat hepatocytes with Li^+. However, both studies clearly establish that EGF induces PIP_2 hydrolysis in liver-derived cells, in contrast to an earlier study in which primary rat hepatocytes were found to be unresponsive [22]. While different methodologies were used, this discrepancy indicates that the ability of primary rat hepatocytes to respond to EGF may vary with hepatocyte preparation. EGF receptor levels in rat hepatocytes, for example, vary depending upon the nutritional state of the animal from which they are isolated [39]. Thus, the PLC/PRF/5 cell line may be a good model system for examining EGF receptor action in liver since EGF receptor activation is still coupled to the inositol phosphate signalling pathway and culture conditions can be rigidly controlled.

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