EGF and insulin action in fibroblasts

Evidence that phosphoinositide hydrolysis is not an essential mitogenic signalling pathway

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Chinese hamster lung fibroblasts (CHL) arrested in G_0 by serum starvation reinitiate DNA synthesis in response to either EGF, thrombin or serum. Arrested cells, prelabelled to equilibrium with [^{3}H]inositol and receiving 20 mM LiCl prior stimulation, released rapidly large amounts of inositol phosphates when stimulated with thrombin or serum. In sharp contrast, EGF alone, or in association with insulin, failed to induce phosphoinositide breakdown at either early or late stages of EGF stimulation or in growing cells in EGF-supplemented serum-free medium. Phospholipase C remained, however, highly activatable by thrombin at all stages of EGF stimulation. Since EGF and thrombin are equally potent mitogens for CHL, we conclude that hydrolysis of polyphosphoinositides is not an exclusive signalling pathway for commitment to DNA replication and cell division.

α-Thrombin *Phosphoinositide cycle* *DNA synthesis* *Fibroblast* *Epidermal growth factor*

1. INTRODUCTION

It is now well documented that a variety of polypeptide growth factors, when added to quiescent cells, initiate rapidly a common set of biochemical events. Among these early and apparently 'ubiquitous' events are stimulation of an Na^+/H^+ antiporter with a subsequent rise in pH_i [1,2], stimulation of an $Na^+/K^+/Cl^-$ cotransporter [3], increase in cytoplasmic Ca^{2+} [4], phosphorylation of ribosomal protein S6 [5,6], and increased expression of proto-oncogenes c-*myc* and c-*fos* [7–9]. How this common pleiotypic response is initiated from the growth factor receptor interaction and whether the secondary messengers are generated by a unique transducing pathway are among the most intriguing questions which are still unresolved.

Here, we have analyzed two equally potent growth factors, α-thrombin and epidermal growth factor (EGF) for reinitiation of DNA synthesis in Chinese hamster lung fibroblasts (CHL). We showed that the first action of α-thrombin on G_0-arrested cells is the hydrolysis of polyphosphoinositides [10], a well-known source of secondary messengers [11,12]. In contrast, EGF alone or together with insulin mediates early and late mitogenic events without activating inositol lipid breakdown. This result strongly suggests that the polyphosphoinositide signalling pathway is not essential to set in motion the G_0/G_1 to S-phase transition in fibroblasts and that additional transducing pathways must exist.

2. MATERIALS AND METHODS

2.1. *Materials*

Highly purified α-thrombin (2660 NIH units/mg) was generously provided by Dr J.W. Fenton II (New York State Department of Health, Albany, NY). Mouse EGF was purified in this laboratory according to Savage and Cohen [13].

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Crystalline bovine insulin was purchased from Sigma. [*methyl*-^{3}H]Thymidine and *myo*-[2-^{3}H(n)]-inositol were from NEN, France.

2.2. *Cell culture*

Secondary cultures of Chinese hamster lung fibroblasts were issued from dissected and trypsinized newborn animal's lung and maintained in DME medium supplemented with 5% fetal calf serum (FCS), penicillin (50 U/ml) and streptomycin (50 μg/ml) in a humidified atmosphere of 5% CO_2/95% air. Experiments were conducted with cells taken after 5–10 passages.

2.3. *DNA synthesis*

Reinitiation of DNA synthesis was measured in confluent cells arrested in G_0 by a 24 h incubation in serum-free DME medium [14]. Cells were then incubated with mitogens for 24 h in DME/Ham's F12 medium (1:1) containing [^{3}H]thymidine (3 μM, 2 μCi/ml). They were then rinsed, fixed with 5% trichloroacetic acid and the radioactivity incorporated assayed after solubilization of the cell extracts in 0.1 N NaOH. Determination of the percentage of labelled nuclei during the 24 h of stimulation with mitogens was performed by autoradiography as in [15].

2.4. *Phosphoinositide hydrolysis*

Confluent cells grown in 35-mm dishes were labelled to equilibrium with [^{3}H]inositol (2 μCi/ml) for 20 h in serum-free DME medium, the period required to bring the cell population to G_0. Cells were then washed with a Hepes-buffered solution containing 20 mM LiCl (110 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$ and 20 mM Hepes/NaOH, pH 7.4) and incubated for 30 min in this solution before addition of growth factor. The hydrolysis of phosphoinositides was followed by measuring the rate of inositol phosphate released, essentially as in [10,16].

3. RESULTS

Secondary cultures of CHL fibroblasts arrested in G_0 by serum starvation for 24 h can be restimulated to duplicate DNA synthesis and to divide by either EGF, α-thrombin or FCS. Insulin alone is a very weak mitogen but potentiates strongly the EGF or α-thrombin response (fig.1A).

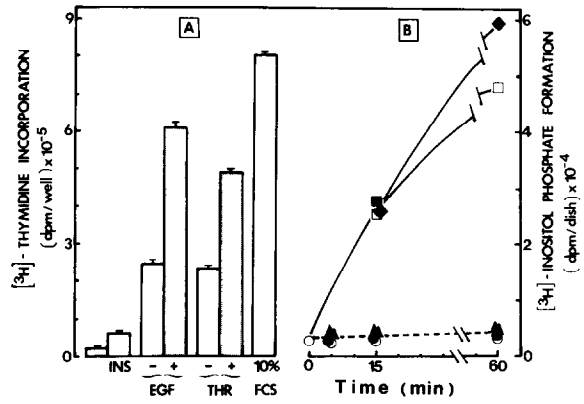

Fig.1. Reinitiation of DNA synthesis and phosphoinositide breakdown in G_0-arrested CHL cells. Confluent cultures of CHL cells were arrested in G_0 by serum starvation as indicated in section 2. (A) Thymidine incorporation measured after 24 h stimulation by different mitogens: insulin, 10 μg/ml (INS); EGF, 10 ng/ml; α-thrombin, 1 U/ml (THR); 10% FCS. Incubations were performed in the absence (−) or presence (+) of insulin. (B) Time course of inositol phosphate formation. [^{3}H]Inositol-prelabelled cells were incubated for 30 min with 20 mM LiCl prior to stimulation with insulin (△), EGF (●), EGF + INS (▲), THR (□), THR + INS (■), FCS (◆) at the same concentrations as in A; no addition (○). Total inositol phosphates (mono-, bis- and trisphosphate) released were measured as reported in section 2. Results are the mean of duplicate dishes.

We have recently reported that a rapidly detectable biochemical action of α-thrombin is the hydrolysis of polyphosphoinositides [10,17]. Increase in inositol trisphosphate content is detected within 5 s of thrombin action. Here we showed that addition of 20 mM LiCl, an inhibitor of inositol monophosphatase [18], had no effect on the rate of inositol phosphate formation. This rate was not measurable over a 60 min period in quiescent cells despite the high sensitivity of the assay (fig.1B). Treatment of the cells with 1 U/ml of α-thrombin or 10% FCS resulted in a rapid and progressive accumulation of inositol phosphates. In contrast, addition of either insulin or EGF or both together had no detectable effect on phosphoinositide hydrolysis suggesting that, under this mitogenic stimulation, phospholipase C is as 'quiescent' as in unstimulated cells. We have seen that insulin can stimulate by 2–3-fold the mitogenic response of α-thrombin (fig.1A [19]). This potentiating action

does not occur at the level of phospholipase C activation, since insulin failed to modify the rate of inositol phosphate release when added at maximal or submaximal concentration (0.01 U/ml) of thrombin (not shown). The percentages of labelled nuclei were determined to compare directly the respective mitogenic potential of the different mitogens used. EGF used at a maximally efficient concentration (100 ng/ml) induced DNA replication of 43% of the cells, a response higher than that observed with 1 U/ml of thrombin (not shown) and nearly equivalent to that observed with 10% FCS (fig.2A). Even under this condition, EGF did not induce the formation of inositol phosphates (fig.2B).

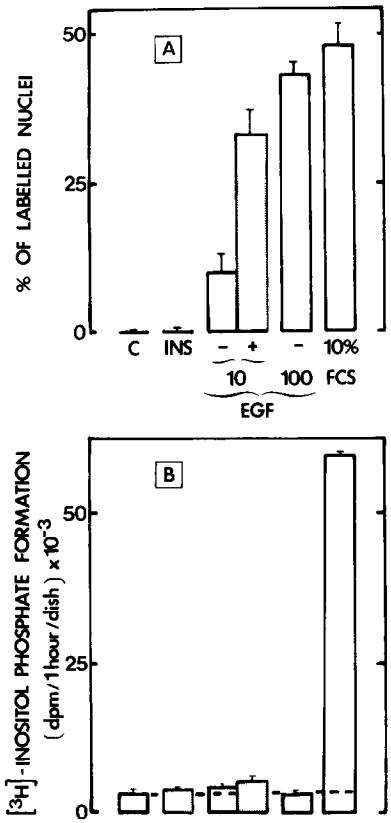

Fig.2. Percentage of cells reinitiating DNA synthesis and phosphoinositide breakdown in response to different stimuli. (A) Arrested CHL cells were stimulated for 24 h by different growth factors: insulin, EGF at 10 or 100 ng/ml in the absence (−) or presence (+) of insulin, 10% FCS, no addition (C). The percentage of labelled nuclei was determined by autoradiography. (B) Inositol phosphates released after 1 h stimulation in the absence of growth factor.

So far we have clearly shown that EGF and insulin do not stimulate inositol lipid breakdown at the early stage of stimulation of G_0-arrested cells. To rule out completely the involvement of this transduction pathway in the mechanism of EGF action it was essential to explore it in cells at the late stage of the G_0/G_1 transit as well as in the exponential growing phase. Fig.3 shows the rate of inositol phosphate formation in CHL cells stimulated with 100 ng/ml of EGF for different time periods: 0 h (A), 8 h EGF-stimulated (B), and EGF-growing cells for 48 h (C). As reported above, the assay has been conducted in the presence of the phosphoinositide cycle blocker (LiCl) to amplify the detection of phospholipase C activation. Regardless of the conditions used, EGF clearly failed to induce the formation of inositol phosphates. This absence of response to EGF cannot be attributed to a lack of phosphoinositide pool since addition of α-thrombin to EGF-stimulated cells resulted in a rapid and massive

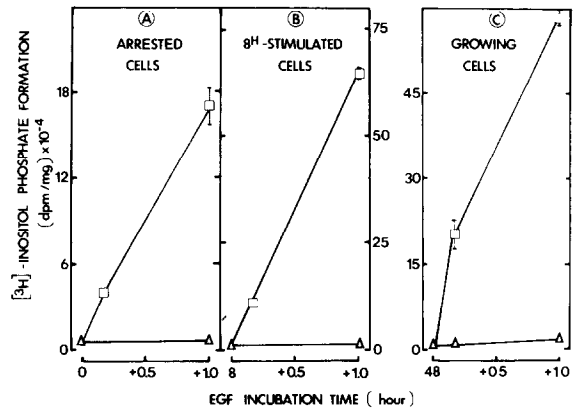

Fig.3. Time course of inositol phosphate release in response to EGF or thrombin in EGF-stimulated CHL cells. [^{3}H]Inositol-prelabelled CHL cells were either arrested in G_0 by serum starvation (A,B) or grown for 48 h in serum-free medium containing EGF, insulin and transferrin (C). Determination of inositol phosphate content was as follows: 20 mM LiCl was added to the culture medium 15 min before adding 100 ng/ml EGF (△) or 0.01 U/ml α-thrombin (□). Total inositol phosphates was determined at 0, 15 and 60 min after stimulation in arrested cells (A), in 8 h EGF-stimulated cells (B) and in EGF-growing cells (C). Points represent the mean value of duplicate experiments.

production of inositol phosphates which proceeded at almost a constant rate over 1 h of stimulation (fig.3A–C).

4. DISCUSSION

Our previous studies on the mechanism of action of growth factors have been more specifically carried out with CCL39, an established cell line of CHL [20]. Because of the poor mitogenic response of G_0-arrested CCL39 cells to EGF, we have chosen here secondary cultures of CHL fibroblasts. Indeed, a direct comparison of the effects of EGF and α-thrombin performed in these cells was more valid since the two growth factors were equally potent not only for reinitiation of DNA synthesis but also for supporting growth in serum-free medium. In this report, we have shown that: (i) unlike α-thrombin or serum, EGF does not stimulate the breakdown of phosphoinositides measured with the LiCl-amplified assay; (ii) the lack of response to EGF occurs at all stages of the cell cycle (early, mid, late G_0/G_1 transit and exponential phase); and (iii) the mitogenic potentiating effect generally observed with insulin does not take place at the level of phospholipase C activation. An important point of this study is that EGF-stimulated cells have kept their potential to initiate phosphoinositide breakdown in response to thrombin (fig.3). However, one might argue that two cell types, one responding to EGF and the other to thrombin, coexisted in the secondary cultures of CHL. This possibility can be ruled out since we observed the same results with a clonal population of CHL (not shown).

Whether EGF is capable of stimulating phosphatidylinositol turnover in other cell types is a matter of controversy. This was reported to be the case in A431 cells [21] in which EGF is a growth inhibitor. In these cells EGF rapidly stimulated the incorporation of $^{32}P_i$ into phosphatidic acid; however, a direct stimulation of phosphoinositide hydrolysis has not been demonstrated. In contrast, and fully consistent with our results, Macphee et al. [22] have shown an increase in phosphatidylinositol turnover in resting Swiss 3T3 cells stimulated by prostaglandin $F_{2\alpha}$ but not by EGF. Such a lack of effect of EGF was recently confirmed by Hesketh et al. [23].

In conclusion, our results firmly establish that EGF alone or in combination with insulin is capable of stimulating G_0-arrested CHL cells to replicate DNA synthesis and to divide without activating phosphatidylinositol turnover. This property is not unique to EGF; we recently observed that pure fibroblast growth factor (basic and acidic forms of FGF) can initiate early events in CHL cells (e.g. rise in cytoplasmic pH, and Ca^{2+}, phosphorylation of S6) and DNA replication without any detectable induction of phosphoinositide breakdown (Moenner et al., in preparation). Consistent with this finding is the fact that the EGF- and FGF-induced cytoplasmic rise in Ca^{2+} is dependent upon the presence of external Ca^{2+}. In contrast, the α-thrombin-induced rise in Ca^{2+} results from mobilization of internal Ca^{2+} stores, presumably as a consequence of α-thrombin inositol trisphosphate formation ([10,11]; I. Magnaldo, unpublished).

It is interesting that evidence in favor of separate growth factor transducing pathways, which converge very early to stimulate the pleiotypic response and DNA synthesis, is emerging. An increasing number of growth-promoting agents (e.g. PDGF [24], α-thrombin [10], bombesin [25], bradykinin, vasopressin [26,27]) appear to initiate their action through the activation of polyphosphoinositide breakdown. Little is known about the mechanism of activation of this system. However, the existence of alternative growth-signalling pathways should help in isolation of inositol lipid cycle-deficient mutants. This might provide a promising approach to analyze at the molecular level the coupling mechanism between growth factor receptor and phospholipase C.

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