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Transmembrane signalling at epidermal growth factor receptors overexpressed in NIH 3T3 cells

Phosphoinositide hydrolysis, cytosolic Ca^{2+} increase and alkalinization correlate with epidermal-growth-factor-induced cell proliferation

Atanasio PANDIELLA,* Laura BEGUINOT,† Thierry J. VELU‡ and Jacopo MELDOLESI*§

*Department of Pharmacology, C.N.R. Center of Cytopharmacology, Scientific Institute S. Raffaele, University of Milano, via Olgettina 60, 20132 Milano, Italy, †University Institute of Microbiology, 1353 Copenhagen K, Denmark, and ‡Laboratory of Cellular Oncology, National Cancer Institute, Bethesda, MD 20892, U.S.A.

NIH 3T3 cells, which express a small number of EGF (epidermal growth factor) receptors, are poorly responsive to EGF. However, when the same cells overexpress the cloned human EGF receptor (EGFR T17 cells), they display EGF-dependent transformation. In EGFR T17 cells (but not in the parental NIH 3T3 cells), EGF is shown here to trigger polyphosphoinositide hydrolysis as well as the generation of the ensuing intracellular signals, the increase in the cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) and pH. EGF induced a large accumulation of inositol 1,4,5-trisphosphate, with a peak at 15–30 s and a slow decline thereafter. Other inositol phosphates (1,3,4-trisphosphate and 1,3,4,5-tetrakisphosphate) increased less rapidly and to a lesser degree. $[Ca^{2+}]_i$ increased after a short lag, reached a peak at 25 s and remained elevated for several minutes. By use of incubation media with and without Ca^{2+}, the initial phase of the EGF-induced $[Ca^{2+}]_i$ increase was shown to be due largely to Ca^{2+} release from intracellular stores. In contrast with previous observations in human A431 cells, the concentration-dependence of the EGF-triggered $[Ca^{2+}]_i$ increase in EGFR T17 cells paralleled that of [^{3}H]thymidine incorporation. It is concluded that polyphosphoinositide hydrolysis, $[Ca^{2+}]_i$ increase and cytoplasmic alkalinization are part of the spectrum of intracellular signals generated by the activation of one single EGF receptor type. These processes might be triggered by the receptor via activation of the intrinsic tyrosine kinase activity. Large stimulation of DNA synthesis and proliferation by EGF in EGFR T17 cells could be due to a synergistic interplay between the two signal pathways initiated by tyrosine phosphorylation and polyphosphoinositide hydrolysis.

INTRODUCTION

Activation of epidermal growth factor receptors (EGFR) has been reported to induce a number of early intracellular events. These include activation of the intrinsic tyrosine kinase activity with phosphorylation of the receptor itself and other protein substrates (Carpenter, 1987; Schlessinger, 1986; Hunter & Cooper, 1985), stimulation of polyphosphoinositide (PPI) hydrolysis (Hepler *et al.*, 1987; Wahl *et al.*, 1987; Pike & Eakes, 1987; Pandiella *et al.*, 1987*a*), increase of cytosolic pH (pH_i) and Ca^{2+} concentration ($[Ca^{2+}]_i$) (Hesketh *et al.*, 1985; Moolenaar, 1986; Hepler *et al.*, 1987; Pandiella *et al.*, 1987*a*). Evidence consistent with their involvement in the stimulation of cell proliferation has been reported for all of these events (Rozengurt, 1986). For EGF, however, the role of PPI hydrolysis, $[Ca^{2+}]_i$ and pH_i is still open to question. In fact, clear changes of these parameters have been observed primarily in the cell types that express large numbers (10^5–10^6/cell) of EGFR, in particular in the human carcinoma cell line A431 (Moolenaar *et al.*, 1986; Pandiella *et al.*, 1987*a*). In addition, the concentrations of EGF that are required to observe these effects induce growth inhibition rather than stimulation of A431 cells (Gill & Lazar, 1981; Barnes, 1982; Kawamoto *et al.*, 1983; Pandiella *et al.*, 1987*b*).

An important tool that has recently become available for the study of EGF physiology is NIH 3T3 fibroblasts (a cell line with only few endogenous EGFR) in which the cloned functional human EGFR has been overexpressed (Velu *et al.*, 1987; Di Fiore *et al.*, 1987). In particular, the EGFR T17 cells isolated by Velu *et al.* (1987) and by T. J. Velu, L. Beguinot, W. C. Vass, K. Zhang, I. Pastan & D. R. Lowy (unpublished work) (EGFR complement $> 3 \times 10^5$/cell) when incubated in the absence of EGF grow similarly to the parental NIH 3T3 cells, whereas in the presence of EGF they display a fully transformed phenotype. We have studied the effect of EGF on PPI hydrolysis, $[Ca^{2+}]_i$ and pH_i in EGFR T17 cells, and found that in EGFR T17 cells, but not in the parental NIH 3T3 fibroblasts, EGF triggers the genera-

Abbreviations used: EGF, epidermal growth factor; EGFR, EGF receptor; PPI, polyphosphoinositide; pH_i, intracellular pH; $[Ca^{2+}]_i$, free cytosolic Ca^{2+} concentration; KRH, Krebs–Ringer medium buffered with Hepes; BCECF, bis(carboxyethyl)carboxyfluorescein; FCS, fetal-calf serum; PDGF, platelet-derived growth factor; DMEM, Dulbecco's modification of Eagle's medium; BME, basal medium of Eagle; Ins*P*s, inositol phosphates; Ins(1,4,5)P_3, inositol 1,4,5-trisphosphate; Ins(1,3,4)P_3, inositol 1,3,4-trisphosphate; Ins(1,3,4,5)P_4, inositol 1,3,4,5-tetrakisphosphate.

§ To whom reprint requests should be addressed.

tion of these three early events at concentrations similar to those needed to stimulate [^{3}H]thymidine incorporation into DNA.

MATERIALS AND METHODS

Cell cultures

NIH 3T3 fibroblasts [wild-type and EGFR T17; for details see Velu *et al.* (1987)] were grown in DMEM containing 10% (v/v) FCS, with penicillin (100 units/ml) and streptomycin (100 μg/ml).

DNA synthesis

Cells seeded in 24-well dishes at 5×10^4 cells/well (Livneh *et al.*, 1986) were grown for 3 days and then switched to serum-free DMEM either with or without various concentrations of EGF; 20 h later [^{3}H]thymidine (5 μCi/well) was added, and the incubation continued for 4 h more, after which wells were washed three times with cold phosphate-buffered saline (Pandiella *et al.*, 1987*a*), and 500 μl of ice-cold 7.5% (v/v) trichloroacetic acid was finally added. After 30 min on ice, the wells were washed with 2×1 ml of ethanol, and the DNA was solubilized with 600 μl of 0.1 M-NaOH. A 500 μl portion of the latter was mixed with 4 ml of NEN Atomlight, and radioactivity counted in a Beckman L30 scintillation spectrometer.

Inositol phosphate assay

Generation of inositol phosphates (Ins*P*s) was investigated in cell monolayers long-term-labelled with *myo*-[^{3}H]inositol. For analysis of total Ins*P*s generation, confluent monolayers in 35 mm-diam. dishes were incubated for 24 h in serum- and inositol-free basal medium of Eagle (BME) supplemented with *myo*-[^{3}H]inositol (5 μCi/ml), then washed twice in a saline medium (KRH) containing (mM): NaCl, 125; KCl, 6; $MgSO_4$ and KH_2PO_4, 1.2; $CaCl_2$, 2; glucose, 6; Hepes/NaOH buffer, pH 7.4, 25. The monolayers were preincubated for 15 min with KRH supplemented with LiCl (10 mM) before addition of EGF. After incubation for a further 10 min at 37 °C, the medium was aspirated and the reaction quenched by addition of 1 ml of ice-cold 7.5% trichloroacetic acid. A 0.9 ml portion of the acid extract was washed with 5×1 ml of diethyl ether, and applied to small (1 ml) Dowex 1X8 (200–400 mesh) anion-exchange columns. After washing with 3 ml of 5 mM-$Na_2B_4O_7$/60 mM-sodium formate, Ins*P*s were eluted with 0.1 M-formic acid/1 M-ammonium formate (Berridge *et al.*, 1983).

For analysis of individual Ins*P*s, confluent monolayers in 60 mm-diam. dishes were labelled as above, but with 10 μCi of *myo*-[^{3}H]inositol/ml, washed three times with KRH and preincubated with or without LiCl. Acid extracts washed with ether were frozen until analysis by h.p.l.c. on a Partisil SAX anion-exchange column. Two protocols were used. The entire spectrum of Ins*P*s was separated as described by Irvine *et al.* (1985, 1986). In brief, the column was first washed with water (6 min) to remove free *myo*-[^{3}H]inositol, and then a step ammonium formate gradient was applied. The salt concentration was first raised linearly to 0.75 M (24 min), then kept constant for 2 min, followed by a second linear increase (to 1 M, 6 min), a 5 min step at this concentration, another linear increase to 1.7 M salt (10 min) and a final step (6 min). Between successive runs, columns were washed for 10 min with water. Throughout the experiments the flow rate was 1.25 ml/min, and fractions were collected at 0.5 min intervals. To separate InsP_3 isomers and InsP_4 only, the protocol described by Batty *et al.* (1985) was used. The flow rate was 2 ml/min, and fractions were collected at 0.33 min intervals. All the samples and [^{3}H]Ins*P*s standards were injected after mixing with a fixed sample of ATP (50 μl of 1 M solution) to serve as an internal standard, revealed by u.v. adsorption at 254 nm. Radioactivity was measured after mixing the fractions with Packard Hionicfluor.

Table 1. Generation of inositol phosphates in EGFR T17 cells

EGFR T17 cells were labelled with 5 μCi of *myo*-[^{3}H]-inositol/ml in serum-free BME for 24 h. The monolayers were washed twice with KRH containing 10 mM-LiCl and preincubated for 15 min in this medium. Thereafter vehicle, EGF (50 nM) or FCS (10%) was added and incubation prolonged for 10 min at 37 °C. Incubations were terminated by the addition of 7.5% trichloroacetic acid, and Ins*P*s were analysed as described in the Materials and methods section. Values are means ± S.D. ($n = 3$).

Treatment	Ins*P*s (c.p.m./dish)
–	14090 ± 775
EGF	23903 ± 4616
FCS	73904 ± 22013

$[Ca^{2+}]_i$ and pH_i measurements with fluorescent probes

Measurements were carried out on cell monolayers as previously described (Pandiella *et al.*, 1987*a*). In brief, confluent monolayers attached to glass coverslips were serum-starved for 24 h and then loaded with fluorescent probes for 45 min at 37 °C. For $[Ca^{2+}]_i$, cells were loaded by incubation with fura-2 acetoxymethyl ester (5 μM in DMEM), then washed twice with KRH and placed in a custom-made holder that was inserted in a 1 cm glass cuvette, equipped with constant stirring and temperature control, containing KRH or the same medium without Ca^{2+} added. Fluorescence was excited at two wavelengths (345 and 380 nm), with emission at 490 nm, measured with a Perkin–Elmer LS-5 spectrofluorimeter. Calibration of $[Ca^{2+}]_i$ signals was made from the 345/380 nM fluorescence ratios, based on the equation proposed by Grynkiewicz *et al.* (1985). In the experimental conditions used, the values in the equation were found to be as follows: $S_{f2}/S_{b2} = 7.0 \pm 0.4$, $F_{min.345}/F_{min.380} = 1.24 \pm 0.4$, $F_{max.345}/F_{max.380} = 20.7 \pm 1.2$ (all means ± S.D.; $n = 4$). Results were carefully corrected for autofluorescence, measured in parallel monolayers, whose contribution never exceeded 10% of total fluorescence. The procedure used for measuring pH_i (Rink *et al.*, 1982) was similar to that for $[Ca^{2+}]_i$, but monolayers were loaded with bis(carboxyethyl)carboxyfluorescein acetoxymethyl ester (BCECF/AM) (6 μM), and fluorescence was excited at 490 nm, with emission at 530 nm. Excitation and emission slits were 5 nm in all measurements.

Materials

Products for cell culture were obtained from Flow Laboratories, Milan, Italy. [^{3}H]Thymidine, *myo*-[^{3}H]-

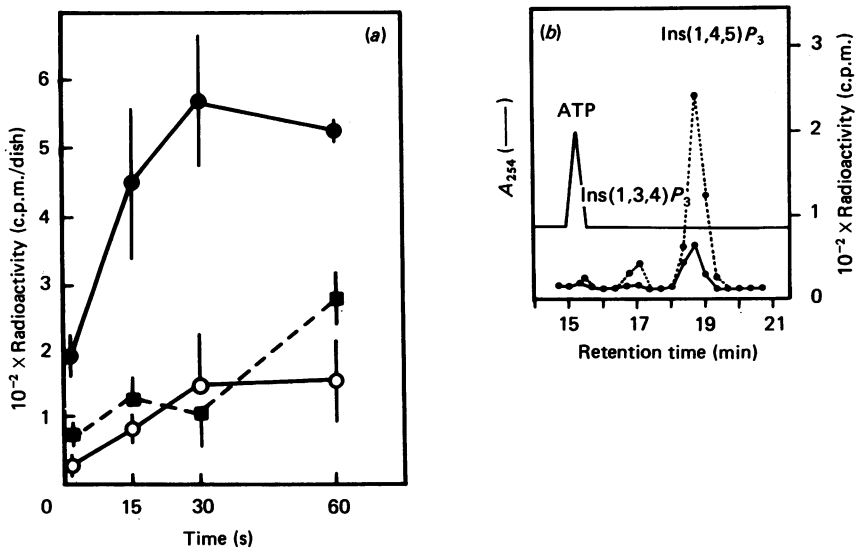

Fig. 1. EGF-induced inositol polyphosphate accumulation in EGFR T17 cells

(*a*) Time course of accumulation of Ins(1,4,5)P_3 (●), Ins(1,3,4)P_3 (○) and Ins(1,3,4,5)P_4 (■) in EGFR T17 cells treated with EGF. Monolayers labelled for 24 h with *myo*-[^{3}H]inositol (10 μCi/ml) were preincubated in KRH medium (without LiCl) for 10 min and then treated with EGF (50 nM) at zero time. Trichloroacetic acid extracts prepared at the indicated time points were processed and then analysed by h.p.l.c. (Batty *et al.*, 1985). Values are means ± S.D. for three experiments. (*b*) Radioactivity profile in the InsP_3 region of the chromatogram from cell extracts prepared before (●——●) and 30 s after (●· · · ·●) addition of EGF. The absorbance peak marks the elution of ATP.

inositol, [^{3}H]Ins(1,4,5)P_3 and [^{3}H]Ins(1,3,4,5)P_4 were from Amersham International; reagents for Ins*P*s measurement were from Fluka; the Partisil SAX h.p.l.c. column was from Whatman; acetoxymethyl esters of fluorescent probes fura-2 and BCECF were from Calbiochem; EGF (receptor grade) was from Collaborative Research; all other chemicals were reagent grade, and were purchased where indicated by Pandiella *et al.* (1987*a*).

RESULTS

Phosphoinositide hydrolysis

Accumulation of radioactive Ins*P*s (the hydrophilic products of PPI hydrolysis) was investigated in wild-type NIH 3T3 cells and in the EGFR T17 cells (EGFR complements $< 10^4$ and $> 3 \times 10^5$/cell respectively; Velu *et al.*, 1987) prelabelled with *myo*-[^{3}H]inositol. Table 1 summarizes the results of initial experiments in which accumulation of total Ins*P*s was measured after a 10 min incubation with 50 nM-EGF in the presence of 10 mM-LiCl, a blocker of inositol-1-phosphatase, used to cause the metabolic trapping of the released Ins*P*s (Berridge *et al.*, 1983). EGF caused a marked increase (+69%) in Ins*P*s accumulation in the EGFR T17 cells, and an even greater increase was induced by FCS, which contains a cocktail of growth and other stimulatory factors. In contrast, no effect of EGF was observed in the parental NIH 3T3 cells (results not shown). In order to identify the nature of the released Ins*P*s, and therefore the nature of the phosphoinositides that serve as substrates of the hydrolytic reaction, h.p.l.c. analyses (Irvine *et al.*, 1985, 1986) were carried out on trichloroacetic acid extracts of EGFR T17 cells. InsP_3 isomers, i.e. Ins(1,4,5)P_3 and Ins(1,3,4)P_3, as well as Ins(1,3,4,5)P_4 were identified by co-elution with reference standards. Ins(1,4,5)P_3, known to cause release of Ca^{2+} from intracellular stores (Berridge & Irvine, 1984; Berridge, 1987) was present in a low amount in untreated EGFR T17 cells, but increased markedly after EGF addition (Fig. 1*a*). The maximum (3-fold over basal) was reached between 15 and 30 s, and was followed by a decline to values only slightly above basal (at 2 and 5 min; results not shown). Ins(1,3,4,5)P_4 and Ins(1,3,4)P_3, the products of the successive phosphorylation and dephosphorylation of Ins(1,4,5)P_3 (Irvine *et al.*, 1986; Hawkins *et al.*, 1986), were hardly detectable in unstimulated cells, and increased progressively up to 1 min after EGF (Fig. 1*a*). In addition to highly phosphorylated Ins*P*s, EGF was able to cause in EGFR T17 cells an increase in inositol bisphosphate (peak at 1 min) and in two inositol monophosphate isomers, which continued to increased for the whole length of the experiment (5 min; results not shown).

$[Ca^{2+}]_i$ increase, cytoplasmic alkalinization and cell growth

As with PPI hydrolysis, EGF was found to have profound effects on $[Ca^{2+}]_i$ and pH_i in EGFR T17 cells, which over-express the human EGFR, but not in the parental NIH 3T3 cells (Figs. 2*a* and 2*b*). Fig. 2(*a*) illustrates the response of EGFR T17 cell monolayers loaded with the fluorescent $[Ca^{2+}]_i$ indicator, fura-2, and then treated with an optimal concentration of EGF (50 nM) in the complete incubation medium (containing 2 mM-Ca^{2+}). Under these conditions $[Ca^{2+}]_i$ began to increase after a short lag (2–5 s), reaching a maximum at 20–25 s. Such a time course matches the corresponding data for Ins(1,4,5)P_3 generation (Fig. 1*a*). On average, the $[Ca^{2+}]_i$ increase was from 95 ± 12 to 206 ± 23 nM (means ± S.D.; $n = 12$ and 7 respectively). Thereafter $[Ca^{2+}]_i$ declined, the average duration of the EGF-induced $[Ca^{2+}]_i$ transient being 5.4 ± 0.6 min ($n = 7$). No EGF effect on $[Ca^{2+}]_i$ was observed in the parental NIH 3T3 cells (Fig. 2*b*). On the other hand, when this same

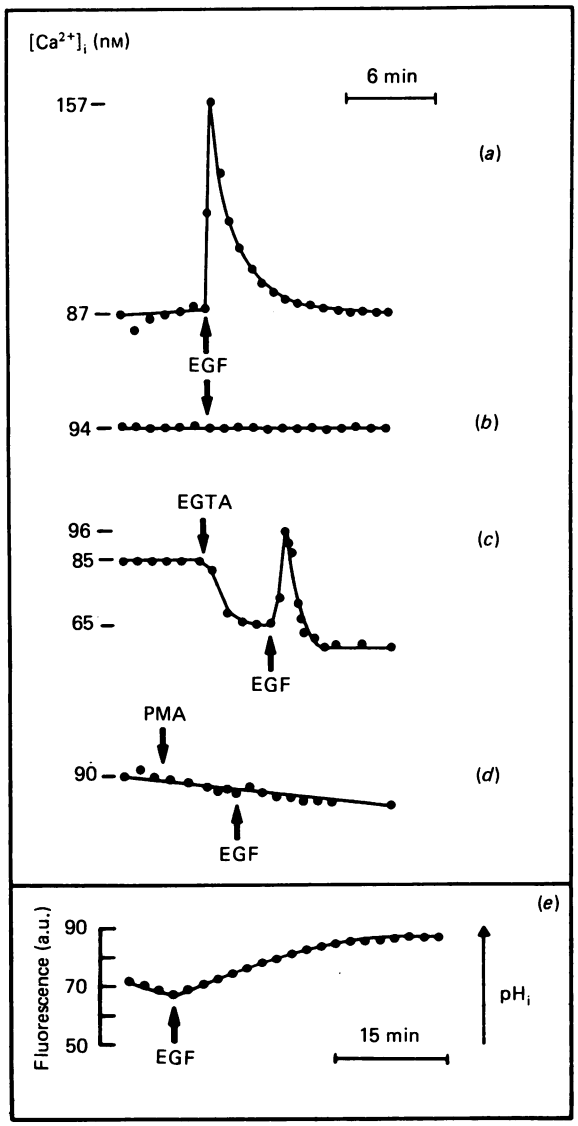

Fig. 2. $[Ca^{2+}]_i$ and pH_i increases induced by EGF in NIH 3T3 and EGFR T17 cells

Monolayers on glass coverslips loaded with either fura-2 (*a–d*) or BCECF (*e*) were analysed in Ca^{2+}-containing (*a*, *b*, *d*, *e*) or Ca^{2+}-free (*c*) KRH medium. Additions marked by arrows were made at the following final concentrations: EGF, 50 nM; PMA (phorbol 12-myristate 13-acetate), 0.1 μM; EGTA, 3 mM. Points in (*a*)–(*d*) are $[Ca^{2+}]_i$ values calculated from F_{345}/F_{380} ratios as recommended by Grynkiewicz *et al.* (1985). Abbreviation: a.u., arbitrary units.

experiment was carried out on EGFR T17 monolayers incubated in a medium with very low Ca^{2+} (< 1 nM; Fig. 2*c*), the initial EGF-induced $[Ca^{2+}]_i$ peak was largely maintained (50–60 %), but $[Ca^{2+}]_i$ fell thereafter, returning to the resting value within 2 min. These results demonstrate that the EGF-induced $[Ca^{2+}]_i$ increase (visible only in the cells overexpressing the EGFR) has a dual origin, intracellular (redistribution from a cytoplasmic store) and extracellular (increased influx).

Stimulation of PPI hydrolysis by EGF is expected to cause the generation of diacylglycerol (Whiteley & Glaser, 1986), and thus to result in the activation of protein kinase C (Nishizuka, 1986). In A431 cells the activation of the latter enzyme is known to induce, on the one hand, a feedback desensitization of the EGFR (Pandiella *et al.*, 1987*b*), and on the other hand, the alkalinization of the cytoplasm, owing to activation of the Na^+/H^+ exchanger. Experiments were carried out to investigate whether these events can also occur in EGFR T17. Fig. 2(*d*) demonstrates that the protein-kinase-C-mediated desensitization is possible, because the $[Ca^{2+}]_i$ increase was no longer seen when EGF was administered to EGFR T17 cells 5 min after treatment with the well-known activator of the enzyme, phorbol 12-myristate 13-acetate. Fig. 2(*e*) illustrates the effect of EGF on pH_i in EGFR T17 cells. A marked but slowly developing alkalinization was observed, beginning at approx. 2 min. Again no effect was observed in wild-type NIH 3T3 cells (results not shown).

A final series of experiments was carried out to compare the concentration-dependence of the EGF effects on cell proliferation and $[Ca^{2+}]_i$. Two sets of monolayers of both NIH 3T3 and EGFR T17 cells were processed in parallel, to be then exposed to various concentrations of the growth factor. One set was loaded with fura-2, and the $[Ca^{2+}]_i$ peak increases occurring in response to EGF additions were measured. The other set was kept either with or without EGF for a total of 24 h with addition of [^{3}H]thymidine during the last 4 h of incubation, and the incorporation of the tracer into the DNA was measured. The results are summarized in Fig. 3. Under the conditions of the experiment, EGF was without effect in the parental NIH 3T3 cells at all concentrations used, whereas in EGFR T17 cells marked dose-dependent stimulations were observed of both [^{3}H]thymidine incorporation (Fig. 3*a*) and $[Ca^{2+}]_i$ peak increase (Fig. 3*b*). Although the concentration-dependence curves for these two effects were not identical, they were clearly in the same concentration range, with maxima around 50 nM, and half-maximal effects at 3–5 nM-EGF. It is worth noting that in these cells the effect of supramaximal concentrations of EGF on growth remained maximally stimulatory, in contrast with the data on A431 cells (Gill & Lazar, 1981; Barnes, 1982; Kawamoto *et al.*, 1983).

DISCUSSION

The results herein reported demonstrate that expression of a high number of EGFR is sufficient to enable a non-tumoral cell line to respond to the application of EGF with a full complement of early intracellular events: stimulation of PPI hydrolysis, with generation of diacylglycerol and Ins*P*s second messengers, followed by increases in pH_i and $[Ca^{2+}]_i$. Our results rule out the possibility that this group of responses is due to a second type of EFGR, structurally different from the well-characterized receptor possessing intrinsic tyrosine kinase activity (Hepler *et al.*, 1987). This conclusion is also supported by the results of two other, less precisely focused, studies (Chen *et al.*, 1987; Pierce *et al.*, 1988). The accumulation of Ins(1,4,5)P_3, the increase in $[Ca^{2+}]_i$ owing to both mobilization from intracellular stores and stimulated influx across the plasmalemma, and the blockade of the $[Ca^{2+}]_i$ response by pretreatment with phorbol esters that we report here in EGFR T17 cells treated with EGF, resemble the effects previously described in the cell model most frequently used for the study of EGF, the human

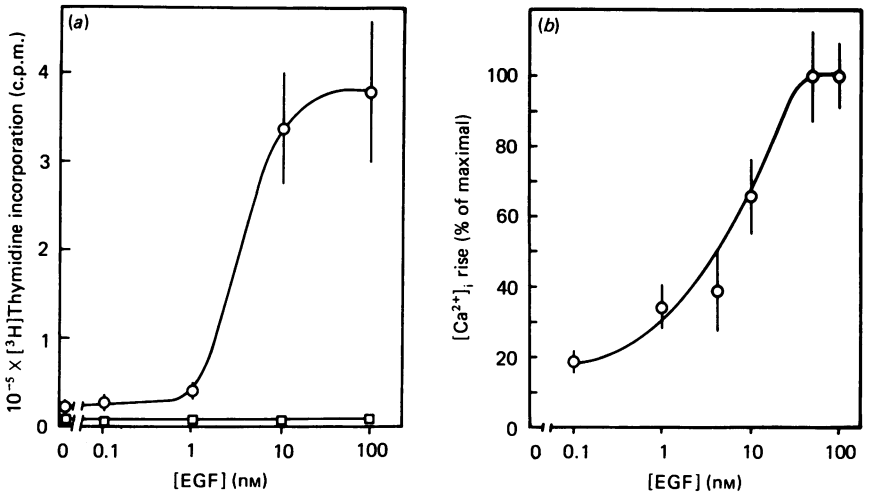

Fig. 3. Concentration-dependence of EGF-induced early ($[Ca^{2+}]_i$ rise) and late ([^{3}H]thymidine incorporation) effects

[^{3}H]Thymidine incorporation (*a*) was measured in cell monolayers cultured in serum-free DMEM for 20 h without or with various concentrations of EGF, before addition of the radioactive marker for 4 h; $[Ca^{2+}]_i$ increases (*b*) are the peak values measured in fura-2-loaded monolayers treated with various concentrations of EGF, expressed as percentages of the value observed with 50 nM-EGF. ○, EGFR T17 cells; □, NIH 3T3 cells. Values shown are means ± S.D. ($n = 3$).

epidermoid carcinoma line A431, which carries a very large number of EGFR (Moolenaar *et al.*, 1986; Hepler *et al.*, 1987; Pandiella *et al.*, 1987*a*; Wahl *et al.*, 1987). Compared with A431 cells, two major differences were found, however, in EGFR T17 cells. On the one hand, Ins(1,3,4,5)P_4 was present in much smaller amount in unstimulated cells, and increased less after EGF addition. On the other hand, the $[Ca^{2+}]_i$ transient elicited by EGF when applied in the Ca^{2+}-containing medium was distinctly shorter-lived. These two differences are probably related, because in different cell systems Ins(1,3,4,5)P_4 [in association with Ins(1,4,5)P_3] has been implicated in the regulation of receptor-triggered Ca^{2+} influx (Irvine & Moor, 1986; Morris *et al.*, 1987), the process responsible for the prolonged phase of the EGF-induced $[Ca^{2+}]_i$ transient. The much larger Ca^{2+} influx previously observed in A431 cells (Moolenaar *et al.*, 1986) might therefore be due to the peculiar metabolism of Ins*P*s in those cells, rather than being a typical effect of EGFR activation.

Stimulation of PPI hydrolysis and $[Ca^{2+}]_i$ increase are events triggered by the activation of a variety of receptors, not only of growth factors, but also of other agents, such as hormones and neurotransmitters (see Berridge, 1987). The $[Ca^{2+}]_i$ increase induced by platelet-derived growth factor (PDGF) in Swiss 3T3 fibroblasts has been found to be initiated more slowly than with most other agents (several seconds delay), and to proceed then at a slower rate (Lopez-Rivas *et al.*, 1987). In EGFR T17 cells we also found slow rates of EGF-induced responses, for both $[Ca^{2+}]_i$ increase and PPI hydrolysis. Especially if one considers the large receptor complement of EGFR T17 cells, this observation appears consistent with the possibility that the mechanism(s) of receptor coupling to PPI hydrolysis are different for EGF and other growth factors on the one hand, and for hormones and neurotransmitters on the other, as might also be predicted from the markedly different structure of the corresponding receptor. Indeed, PPI hydrolysis and $[Ca^{2+}]_i$ increase might not be among the primary events triggered by EGF, but might occur indirectly via the activation of the tyrosine kinase intrinsic to the EGFR molecule (see Chen *et al.*, 1987).

In most non-tumour cell types, including the parental NIH 3T3 cells, EGF is a poor mitogen by itself, but elicits a potentiation, or drives progression in the cell cycle, when combined with another growth factor, such as PDGF (Rozengurt, 1986). However, NIH 3T3 cells are not suitable for biochemical studies of EGF action, because they express too few receptors. On the other hand, in the widely used tumoral cell line, A431, EGF has a marked mitogenic activity only at very low (< 0.1 nM) concentrations. At the high (> 1 nM) concentrations needed to stimulate PPI hydrolysis and $[Ca^{2+}]_i$ increase, EGF is known to trigger not a stimulation, but a slowing down, of A431 growth, ultimately leading to cell death (Gill & Lazar, 1981; Barnes, 1982; Kawamoto *et al.*, 1983). This result was hard to explain, because work with a variety of other mitogens had indicated that PPI hydrolysis and its related events, $[Ca^{2+}]_i$ increase, activation of protein kinase C and cytoplasmic alkalinization, played stimulatory or permissive roles in cell growth (Rozengurt, 1986; Chambard *et al.*, 1987). Our present findings in EGFR T17 fibroblasts, which over-express the normal human EGFR, provide an answer to the problem. These cells are certainly a more physiological model than A431 cells, and express a number of EGFR adequate for biochemical studies. In these cells we find that the concentrations of EGF that induce maximal PPI hydrolysis and $[Ca^{2+}]_i$ increase also maximally stimulate [^{3}H]-thymidine incorporation. From these data, the death of A431 cells in response to nanomolar concentrations of EGF may be a peculiarity of this tumour cell line (synthesis of an endogenous inhibitory factor?), rather than a general toxic effect of the growth factor. The marked mitogenic activity of PDGF, in a variety of cell types, had been previously suggested to be sustained by

the synergistic interplay of two intracellular pathways of stimulation, one dependent on the activation of the receptor-intrinsic tyrosine kinase activity, the other on the stimulation of PPI hydrolysis (Hunter & Cooper, 1985; Rozengurt, 1986). Our present results demonstrate that the ability of EGF to trigger a vigorous proliferation of EGFR T17 cells (Fig. 3; Velu *et al.*, 1987) is sustained by a spectrum of early intracellular signals at least qualitatively similar to that of PDGF. The involvement of a similar synergistic interplay therefore appears likely in the case of EGF too. At present, however, the information about the mechanisms linking the generation of early signals to cell proliferation is still largely conjectural. Because of its well-characterized growth properties, and its ability to acquire transformed behaviour by prolonged treatment with EGF, the EGFR T17 cell model promises to become a useful tool in this field of study.

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