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Evidence for a Growth Effect of Epidermal Growth Factor on MDA-MB-231 Breast Cancer Cells

N. Veber, G. Prévost, P. Planchon and A. Starzec

MDA-MB-231 is a breast epithelial cell line which possesses large amounts of epidermal growth factor (EGF) receptor on its cell surface but does not respond to EGF under standard culture conditions. 8-bromo-cyclic AMP (8Br-cAMP) and cholera-toxin treatments inhibit its growth by increasing its intracellular cAMP level. However, when inhibited in this way, MDA-MB-231 remains unresponsive to EGF. Similar effects—cAMP accumulation and inhibition of cell growth—are produced by forskolin. In addition, this substance specifically blocks MDA-MB-231 cells in G1 phase of the cell cycle. EGF is able to reverse the effect of forskolin on cell proliferation and prevents accumulation of cells in G1 phase without any change of cAMP level. Thus, only when inhibiting cell growth with forskolin does a mitogenic effect of EGF become evident. As cAMP is increased to a similar degree by all three compounds, yet only the effect of forskolin is antagonised by EGF, we suggest that a non-cAMP-mediated effect of forskolin must be considered to explain this effect. In contrast, the mitogenic effect of EGF on the NPM14T4/9 breast epithelial cell line does not change in the presence of forskolin.

Key words: EGF receptor, forskolin, cAMP, breast carcinoma cell line
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INTRODUCTION

EPIDERMAL GROWTH factor (EGF) is potentially mitogenic for a variety of cultured cells, including mammary epithelial cells. It exerts its activity through specific membrane receptors (EGFR) in both normal and tumour cells [1]. In breast tumours, EGFR are found in various amounts [2] and high levels are usually associated with a poor prognosis [3]. That is, primary breast tumours with larger amounts of EGFR are more likely to progress in malignancy than those with lower amounts. Indeed, large amounts of EGFR are more frequently found in lymph node metastases than in primary tumours suggesting that the presence of EGFR may be associated with the metastatic phenotype [4]. Further, tumour relapse appears even more frequently in cases with many EGFR and few oestrogen receptors [5, 6].

Paradoxically, the mitogenic activity of EGF *in vitro* does not appear to necessarily relate to the level of EGFR since EGF fails to stimulate the growth of some human breast carcinoma-derived cell lines with high amounts of EGFR [7]. Such is the case for at least three breast cancer cell lines, MDA-MB-231, BT-20 and Hs578T [8]. However, it appears unlikely that the failure of EGF to stimulate MDA-MB-231 cell proliferation relies on functional defects of EGFR. Indeed, a tyrosine kinase inhibitor (RG-13022) inhibits autophosphorylation of EGFR stimulated by EGF [9]. Moreover, an autocrine loop for EGF and transforming growth factor (TGF)α [10, 11] or TGFα-like protein [12, 13] cannot explain the lack of response of EGF-stimulated MDA-MB-231 cells, since EGFR antibodies do not inhibit MDA-MB-231 growth [14].

The purpose of this study was to ensure the functionality of EGFR in MDA-MB-231 cells. With this aim in view, we attempted to elicit a mitogenic effect of EGF on this cell line by reducing *in vitro* its basal cell growth rate, assuming that high growth rate might overwhelm potential minor effects of EGF. First, the serum concentration present in the culture medium was reduced in order to remove potential proliferative effects of exogenous growth factors. Secondly, cells were treated with various drugs such as 8Br-cAMP, forskolin and cholera toxin which are strong inhibitors of breast cancer cell growth [15, 16]. Using these drugs, we studied the modulation by EGF of cell proliferation, and of the cell cycle in the malignant MDA-MB-231 line. For comparison, studies were also made using the non-malignant NPM14T4/9 line [17, 18] which did proliferate faster in the presence of EGF.

MATERIALS AND METHODS

Cell lines and culture

The human breast carcinoma cell-line MDA-MB-231 was obtained from the American Type Culture Collection. The human breast immortalised cell line NPM14T4/9 was established and characterised in our laboratory [17, 18] and derived from a benign mastopathy. All epithelial cell lines were grown in Dulbecco's modified Eagle's medium (DMEM, Gibco, Grand Island, New York, U.S.A.) supplemented with 1 mM sodium pyruvate (Gibco), 50 U/ml penicillin associated with 50 μg/ml streptomycin (Gibco), 2.5 μg/ml fungizone (Gibco), 10% heat-inactivated fetal calf serum (FCS, IBF), at 37°C in a humidified atmosphere containing 5% CO_2.

Cell proliferation assays

Cells were plated at 2×10^4 cells per well (24-multiwell plates, Falcon) in 1 ml medium. Following cell attachment, 10 ng/ml EGF (Sigma, St Louis, Missouri, U.S.A.), 0.1 mM 8Br-cAMP (Sigma), 0.1 mM forskolin (Calbiochem, San Diego, California, U.S.A.), and 50 nM cholera-toxin (Sigma) were added alone or in combination to the culture medium. At indicated times, cells were washed in a phosphate-buffered saline solution (PBS, Gibco), trypsinised (0.025% trypsin and 0.01% EDTA, Gibco) for 10 min at 37°C and counted in a Coulter counter (Coultronics, Margency, France). All determinations were performed in triplicate. We compared the treatments by an analysis of covariance.

Starzec and colleagues [19] described a dose-dependent inhibition of epithelial cells issued from benign breast diseases and cancer with 8Br-cAMP (50 μM-2mM) and cholera toxin (1–50 nM) treatments. From these results, we selected 100 μM of 8Br-cAMP and 50 nM of cholera toxin for our experiments. We have tested three doses of forskolin on MDA-MB-231 cell-growth. We reduced the proliferation of the cells using 100 μM of forskolin, and selected this dose for further experiments, while this effect was not observed using 1 and 10 μM of the drug (data not shown).

Labelled EGF assays

Iodination of EGF. Mouse EGF (EGF receptor grade, Sigma) was iodinated with [^{125}I]NaI (Amersham, Bucks, U.K.) using the chloramine T method and eluted through a sephadex G-25 column (Pharmacia, Milwaukee, Wisconsin, U.S.A.) as described by Carpenter and colleagues [20]. EGF was iodinated to specific activity ranging from 10^{17} to 10^{18} cpm/mol EGF.

[^{125}I]EGF binding to cell lysates. Cells were plated in 150-mm Petri dishes (Falcon, Becton Dickinson, New Jersey, U.S.A.) in DMEM containing 10% FCS. After 72 h, cells were washed twice with ice-cold PBS, scraped, centrifuged (1000 rpm for 10 min at 4°C) and resuspended in a 10-mM tris–HCl pH 7.2 (Sigma), 20% glycerol (Prolabo, Paris, France), 1 mM phenylmethylsulphonide fluoride (PMSF, Sigma) solution in order to obtain 1-g pellet/ml of buffer. Cells were broken with a Dounce (Type B pestle, 10 strokes) and stored at −80°C. The total protein content of cell homogenates were measured by a Biuret technique (Sigma Diagnostic). For the [^{125}I]EGF binding and cross-linking experiments, cell extracts were diluted in 10 mM Tris–HCl, pH 7.2 and 0.1% bovine serum albumin (BSA, Sigma) at a final concentration of 1 mg protein/ml.

Aliquots of cell lysates (0.1 mg protein/sample) were incubated with 2.4 ng of [^{125}I]EGF and increasing amounts of unlabelled EGF ranging from 1 to 200 times more. The final volume was adjusted to 250 μl by adding a 10-mM Tris-HCl, pH 7.2 and 0.1% BSA solution. The incubation was carried out under equilibrium conditions (2h 30 min at 37°C). Then, 250 μl of 0.1% cold γ-globulin (Sigma) and 500 μl of 25% polyethyleneglycol 6000 (PEC, Merck, Darmstaat, Germany) in 10 mM Tris–HCl, pH 7.2 buffer were added to each sample to precipitate the receptor/EGF complexes. Samples were vortexed, left for 15 min at 4°C and then centrifuged (5000 rpm for 30 min at 4°C). Pellet-associated radioactivity was counted in a γ-counter (LKB, Milwaukee, Wisconsin, U.S.A.).

A similar assay procedure was employed for Scatchard analysis of EGF binding except that aliquots of cells (0.1 mg protein/sample) were incubated with increasing amounts of [^{125}I]EGF (0.2–21 ng) in the absence (total binding) and in the presence (non-specific binding) of a 200-fold excess of unlabelled EGF. Specific binding was calculated from the difference between total and non-specific binding. Apparent Kd and binding site concentrations were calculated according to Scatchard [21].

Covalent cross-linking of [^{125}I]EGF to its receptors. [^{125}I]EGF binding was performed as described above except that [^{125}I]EGF (200 000 cpm) was added to the samples with and without a 200-fold excess of unlabelled EGF. When the binding had reached equilibrium, samples were centrifuged. Cross-linking was carried out by adding 40 μl of 10^{-4} M ethylene glycol-bis (succinimidyl succinate) (EGS, Sigma), 10 mM Tris–HCl, pH 7.2 buffer to the pellets. The reaction was continued for 15 min at 4°C and stopped by addition of 20 μl of loading buffer [0.25 M Tris–HCl, pH 7.2, 50% glycerol, 10% sodium dodecyl

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sulphate (SDS), 0.025% bromophenol blue] and 5 μl of β-mercaptoethanol (Sigma). Proteins were denatured at 95°C. Samples were run on 7.5% polyacrylamide–SDS gels which were autoradiographed for 10 days at −80°C with X-ray films (Amersham).

cAMP assays

Cells were plated at 2×10^5 cells/ml in 24-multiwell plates. After 24 h, cells were treated for 6 h with 0.1 mM 8Br-cAMP, 0.1 mM forskolin, or 50 nM cholera-toxin alone or combined with 10 ng/ml of EGF. Cells were then washed twice with 25 mM ice-cold *N*-(2-hydroxyethyl) piperazine-*N*-(2-ethanesulphonic acid) buffer (HEPES, Gibco) before adding 0.1 N HCl (4°C). Cells were collected by scraping, sonicated and centrifuged (7000 rpm for 15 min at 4°C). Each supernatant was collected (100 μl), mixed with 4 μl of 3 M sodium acetate (Merck) and stored at −80°C. The acetylation of the samples was performed to improve the sensitivity of cAMP assays. Determination of cAMP was performed by using the RIA-NEN cAMP assay protocol (Du Pont, Wilmington, Delaware, U.S.A.) where the competitive binding assay employs [^{125}I]cAMP and a prereacted first and second antibody complex.

Cell cycle analyses by flow cytometry

Cells were plated at 5×10^5 cells/25-cm^2 flask and treated with 10 ng/ml EGF, 0.1 mM 8Br-cAMP, 0.1 mM forskolin and 50 nM cholera toxin, alone or in combination for 48 and 96 h. Cells were washed and harvested. One millilitre of stain solution [5 μg propidium iodide (Sigma), 0.5 mg tri-sodium citrate (Prolabo), 0.25 mg ribonuclease A (Sigma), 0.75 μl Triton X100 1/5 (Sigma)] was added to 10^6 cells for 10 min at 37°C. 0.1 ml of 9.9% NaCl stopped the reaction. The total DNA content per cell was assessed by analysis of fluorescence using a FACScan flow cytometer (Becton Dickinson) 5×10^4 cells/sample were handled. Data were displayed as total number of cells in each of 1024 channels of increasing fluorescence intensity and the resulting histogramme was analysed using the parametric polynomial model.

RESULTS

Effects of EGF on growth of MDA-MB-231 and NPM14T4/9 cells in standard medium

The proliferative effect of EGF was tested on the two cell lines cultured in DMEM supplemented with 10% FCS. Under these conditions, 10 ng/ml of EGF increased the growth rate of NPM14T4/9 markedly ($P \leq 0.01$), resulting in over 80% higher densities after 4 days of treatment (Figure 1), while MDA-MB-231 growth was unaffected ($P = 0.42$). Moreover, neither 1 nor 100 ng/ml of EGF induced any significant changes in MDA-MB-231 cell growth (data not shown).

Characterisation of EGFR on MDA-MB-231 and NPM14T4/9 cell lysates

In order to compare the expression of EGFR on MDA-MB-231 and NPM14T4/9, the EGF-specific binding was evaluated on cell-free lysates. Both cell lines had the same gradual shift of EGFR labelling (Figure 2a). As described by Nicholson and colleagues [22], an estimate of the affinity constant can be derived from the concentration at which the percentage of [^{125}I]EGF specific-binding is 50% (IC_{50}). The derived Kd was in the range of 35 nM. Moreover, both cell lines possessed EGFR quantities of the same order of magnitude; the EGFR amount of MDA-MB-231 was approximately 1.35-fold higher than that of

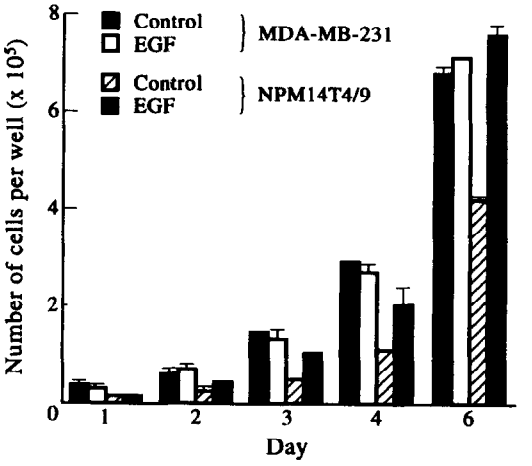
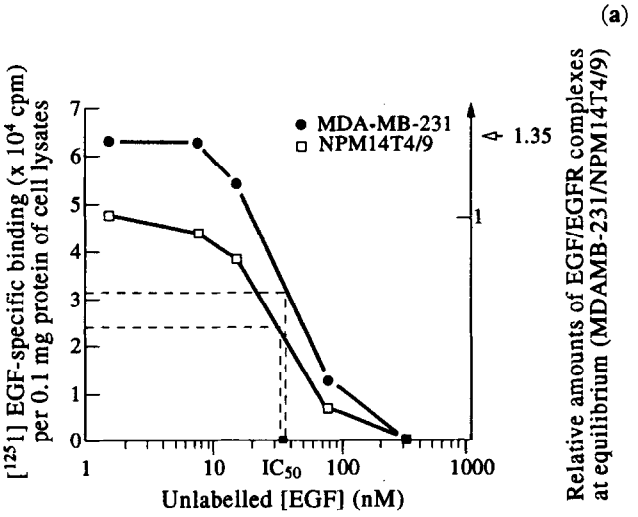


Figure 1. Effect of EGF (10 ng/ml) on growth of NPM14T4/9 and MDA-MB-231 cells cultured in DMEM supplemented with 10% FCS. Values are means ± S.D. calculated from three replicates at each time point.



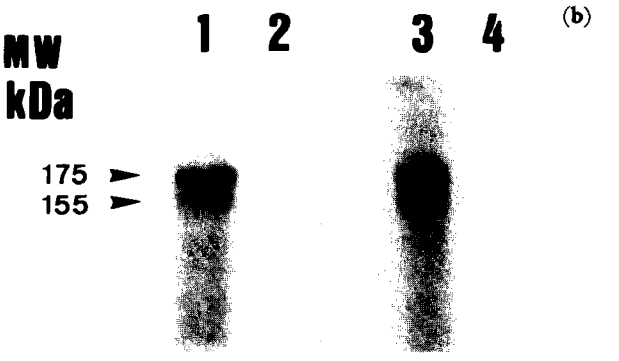


Figure 2. (a) Competitive binding of NPM14T4/9 and MDA-MB-231 cell lysates with 2.4 ng of [^{125}I]EGF and various amounts of unlabelled EGF (ranging from 1 to 200 times more). The EGFR relative amount of MDA-MB-231 cells is evaluated at equilibrium of the EGF binding to the cell lysate (2 h 30 min incubation at 37°C) and is referred to that of NPM14T4/9. (b) [^{125}I]EGF cross-linking experiment: this autoradiogramme shows the binding of [^{125}I]EGF (200 000 cpm) with 0.1 mg of proteins from MDA-MB-231 (lanes 1 and 2) and NPM14T4/9 (lanes 3 and 4) cell lysates. The total binding is presented on lanes 1 and 3 while the non-specific binding obtained with a 200-fold excess of cold EGF is presented on lanes 2 and 4. The molecular weights (MW) of the EGF/EGFR specific complexes are 175 and 155 kDa.

NPM14T4/9. To verify that EGF was bound to the same receptors in both lines, an [^{125}I]EGF cross-linking experiment was carried out. Both lines presented identical receptor/[^{125}I]EGF complexes of 175 000 and 155 000 Daltons (Figure 2b). The formation of these radioactive complexes was totally suppressed by addition of cold competitor, indicating that EGF was specifically bound to the same proteins on MDA-MB-231 and NPM14T4/9 cells.

In summary, the two studied cell lines produced structurally similar EGFR (in terms of size and affinity to the ligand) at similar quantities.

The mitogenic effect of EGF on inhibited MDA-MB-231 and NPM14T4/9 cells

One possible explanation of the absence of EGF effects on the MDA-MB-231 line is that, in the presence of the growth factors provided by the 10% serum, these cells proliferate so rapidly that any additional effect of the EGF would be comparatively too small to measure. To test this hypothesis, cells were cultured in low serum conditions. However, EGF still had no effect at 0.5% serum ($P = 0.81$), a concentration which reduced the cell proliferation rate by over 70% during a 4-day treatment (Figure 3). Thus, the lack of responsiveness of MDA-MB-231 to added EGF did not seem to be due to overwhelming amounts of EGF already in the FCS.

We tried the second alternative which was to reduce the proliferation of the cells using growth inhibitory factors that interfere with cell cycle mechanisms. MDA-MB-231 cell growth was successfully inhibited with various compounds such as phorbol ester (pDBu), purines (ATP, AMP) or staurosporine, but that unfortunately did not reveal an EGF mitogenic effect (data not shown). Therefore, cells were treated with one analogue (8Br-cAMP) and two c-AMP stimulating agents (forskolin, cholera toxin) which were described as strong inhibitors of breast cancer cell growth. 8Br-cAMP (0.1 mM) inhibited MDA-MB-231 ($P \leq 0.01$) and NPM14T4/9 ($P \leq 0.01$) growth by over 88%, compared to the controls, after a 4-day treatment (Figure 4a and 5a). Inhibition caused by forskolin or cholera toxin was weaker than that caused by 8Br-cAMP, although of the same range with respect to each other: MDA-MB-231 growth decreased by 25% and NPM14T4/9 by 60% after 4 days of treatment with forskolin ($P \leq 0.01$; Figures 4b and 5b) or

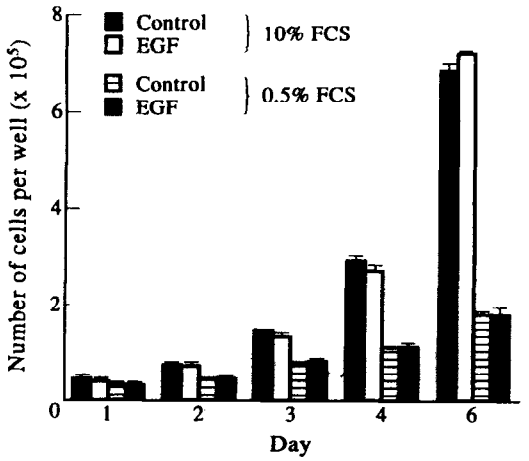


Figure 3. Effect of EGF (10 ng/ml) on growth of MDA-MB-231 cells cultivated in DMEM supplemented with 0.5% FCS; comparison with a 10% FCS supply. Values are means ± S.D. calculated from three replicates at each time point.

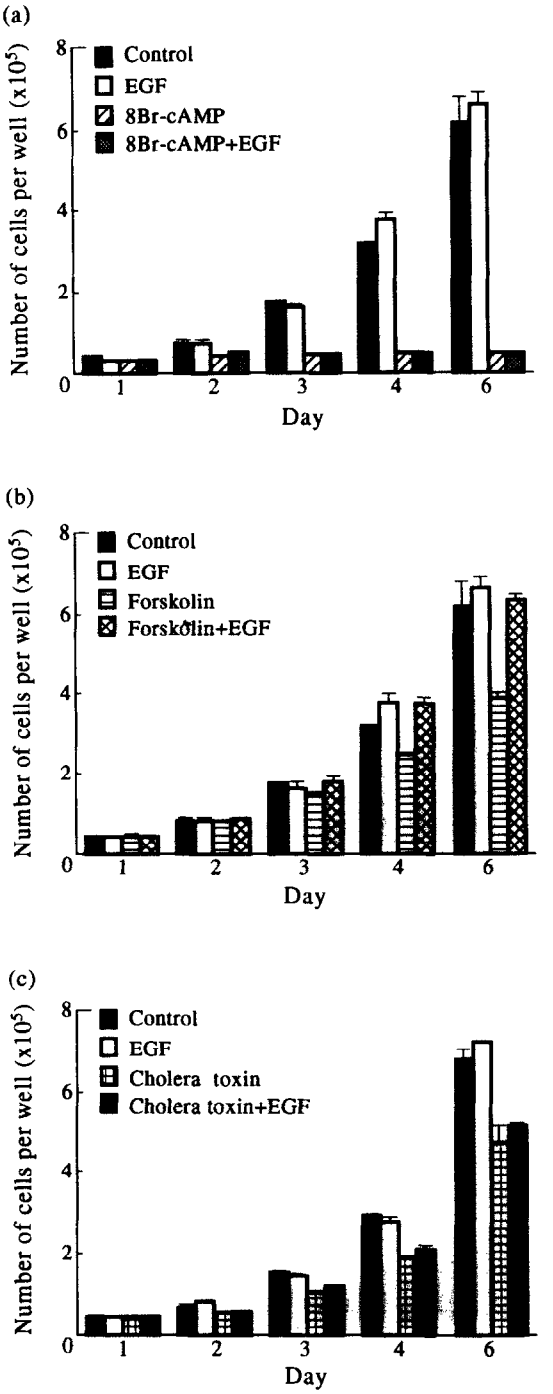


Figure 4. Time-dependent modulations of MDA-MB-231 cell growth with (a) 8Br-cAMP (0.1 mM), (b) forskolin (0.1 mM), or (c) cholera toxin (50 nM), single treatments or combined with EGF (10 ng/ml). Means ± S.D.(/well) of three replicates are shown.

cholera toxin ($P = 0.05$ and $P \leq 0.01$, respectively; Figures 4c and 5c). All three cAMP enhancers inhibited cell growth in a dose-dependent manner (data not shown). NPM14T4/9 cells which were treated so as to increase the intracellular concentration of cAMP responded to EGF in the same way as the control, non-treated NPM14T4/9 cells ($P \leq 0.05$; Figure 5). Note that NPM14T4/9 cells were seeded with 20 000 cells per well in Figure 5c while cells were plated at 12 000 cells per well in Figure 5a and b experiments. Thus, the proliferation of control cells was faster in Figure 5c, and confluence was already

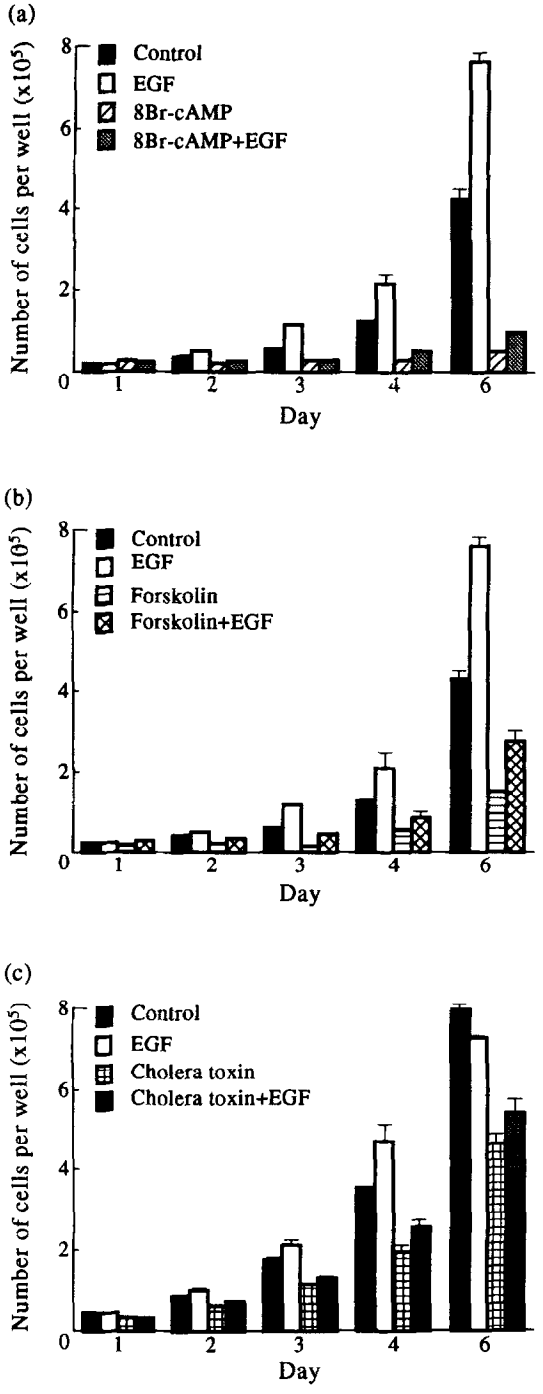


Figure 5. Time-dependent modulations of NPM14T4/9 cell growth with (a) 8Br-cAMP (0.1 mM), (b) forskolin (0.1 mM), or (c) cholera toxin (50 nM), single treatments or combined with EGF (10 ng/ml). Means ± S.D.(/well) of three replicates are shown.

reached on the fifth day. At that time, no EGF growth stimulation can be observed. In this experiment, however, the behaviour of the MDA-MB-231 cell line changed. 8Br-cAMP alone or in combination with EGF (Figure 4a) identically inhibited MDA-MB-231 growth ($P \leq 0.01$), as did cholera toxin compared to cholera toxin plus EGF beyond two days of treatment (Figure 4c). Indeed, no EGF growth effect was observed in the combined treatments with 8Br-cAMP ($P = 0.74$) or with cholera toxin ($P = 0.76$). In forskolin-treated MDA-MB-231 cells, EGF appeared to have a significant stimulatory effect ($P \leq 0.01$; Figure 4b).

The cell number of forskolin-treated cells (Figures 4b and 5b) is significantly increased in the presence of EGF but EGF itself is mitogenic for NPM14T4/9 while it is not for MDA-MB-231. The interaction between forskolin and EGF was analysed using the multiplicative model of Momparler [23]. We noted for NPM14T4/9 that the observed percentage of cell number with forskolin + EGF combined treatment is equal to the percentage of cell number with forskolin treatment multiplied by the percentage of cell number with EGF treatment. Thus, the effects of both agents on NPM14T4/9 cell growth are independent. With regard to MDA-MB-231, the presence of EGF antagonises the inhibitory effect of forskolin. This suggests that EGF requires forskolin to affect MDA-MB-231 growth.

Thus, only by inhibiting MDA-MB-231 cell growth with forskolin, an EGF effect became evident.

Cell cycle analysis

A cell cycle analysis was performed with both cell lines in order to trace other specific effects of forskolin. The cell cycle distribution data were obtained from at least seven independent experiments and were submitted to an analysis of variance. Table 1 presents a typical experiment.

MDA-MB-231. The cell cycle distribution of MDA-MB-231 was greatly changed after a 48 h forskolin-treatment (Table 1). The proportion of cells in the Go/G1 phase increased ($P \leq 0.01$) and, concurrently, the proportion of cells in the S phase decreased ($P = 0.02$); the apparent decrease of the G2 + M phase was not significant ($P = 0.37$). The G1 block caused by forskolin was reversed by EGF ($P \leq 0.05$). The cells reached confluence (high density of the Go/G1 phase sub-population, reduced DNA synthesis, and an almost complete absence of mitosis) on the fourth day of treatment. Thus, the reversion induced by EGF concerning Go/G1 and S phases was no longer perceivable ($P = 0.89$ and $P = 0.92$, respectively). Alternatively, the relatively high proportion of cells in S phase during 8Br-cAMP treatment appeared to be an artefact, due to the limited power of the method. The real synthesis of DNA measured with a bromodeoxyuridine incorporation was found to be in the same order of magnitude as in the control cells (92% of the control, $P = 0.68$).

NPM14T4/9. These cells remained in the exponential growth phase during the 48 and 96 h of treatment. The control cultures without drugs displayed a relatively stable cell cycle distribution over the duration of the experiment (Table 1). The main observed effect was the increase of the percentage of cells in S phase ($P = 0.02$) with time, during EGF exposure. Concurrently, the percentage of cells in G0/G1 phase ($P \leq 0.01$) was decreased. In addition, the proportion of cells in S phase was higher in cultures co-treated with EGF and one drug than in cultures treated with one drug only ($P = 0.01$). The cell cycle distribution also changed when the cells were incubated with 8Br-cAMP. The percentage of cells in the G0/G1 phase increased ($P < 0.01$), while the proportion of cells in S phase ($P \leq 0.01$) and G2 + M phase ($P = 0.03$) decreased with time. This indicates that in NPM14T4/9 cells 8Br-cAMP caused a retardation of progression into S phase or, alternatively, a G1 block in a time-dependent manner.

Effect of forskolin on [^{125}I]EGF binding

Since EGF requires forskolin to affect MDA-MB-231 growth, we thought that forskolin might affect the quantity or the activity of EGFR. EGF binding assays were, therefore, performed with both cell lines after a 48-h forskolin treatment. Due to the

Table 1. Distribution of cells in various phases of cell cycle

Treatments	Cell cycle phases 48 h G0/G1	S	G2 + M	96 h G0/G1	S	G2 + M
Cell line: MDA-MB-231						
Control	47*	34	19	72	22	5
EGF	48	35	17	72	27	1
8Br-cAMP	46	42	13	49	40	11
8Br-cAMP + EGF	43	41	16	50	42	8
Forskolin	81	13	6	80	11	8
Forskolin + EGF	59	27	13	82	11	6
Cholera + toxin	46	39	15	65	24	11
Cholera toxin + EGF	45	35	19	68	22	10
Cell line: NPM14T4/9						
Control	55*	31	13	55	33	12
EGF	47	35	18	44	45	11
8Br-cAMP	65	31	5	70	24	6
8Br-cAMP + EGF	59	37	4	63	29	8
Forskolin	59	23	17	42	37	21
Forskolin + EGF	55	34	11	38	47	15
Cholera toxin	46	37	17	44	38	18
Cholera toxin + EGF	47	34	19	40	49	11

*All values are expressed as percentage of cells in each phase of cell cycle. Cells were cultured in 25-cm² plastic tissue culture flasks and were treated with 0.1 mM 8Br-cAMP, 0.1 mM forskolin, 50 nM cholera toxin and 10 ng/ml EGF alone or in combination for 48 and 96 h. Their DNA was stained with propidium iodide and the fluorescence intensity of 5000 cells per sample was analysed using the parametric polynomial model.

variation in cell confluence with or without treatment, EGFR affinities and capacities were not directly comparable. To standardise the quantity of cell material, binding experiments were performed with cell homogenates. Scatchard analysis of EGF binding indicated curvilinear plots for MDA-MB-231 as well as NPM14T4/9 cells, suggesting two classes of binding sites with intrinsically different affinities (Table 2): one type of high affinity and low capacity,one type of low affinity and high capacity. Analysis of variance of the replicate Kd data and capacity data showed no significant differences between forskolin-treated and non-treated cells ($P > 0.05$).

cAMP mediation

The effects of adenylate cyclase activators and EGF were evaluated on cAMP accumulation. As shown in Table 3, a forskolin 6-h treatment as a cholera toxin treatment enhanced the intracellular cAMP level of both lines. Exogenous 8Br-cAMP permeates the cell membrane and, therefore, increases the cAMP level; this cAMP analogue is identified by the prereacted first antibody of the RIA-NEN kit as intracellular specific cAMP. Indeed, the cAMP levels of NPM14T4/9 and MDA-MB-231 were distinctly higher in the presence of 8Br-cAMP than their respective controls. A forskolin, a cholera toxin or an 8Br-cAMP co-treatment with EGF for the MDA-MB-231 cells did not modify these cAMP observed results (data not shown). A kinetics experiment of cAMP accumulation that was performed on MDA-MB-231-treated cells confirmed the above-described results: (i) forskolin, cholera toxin and 8Br-cAMP enhanced the intracellular cAMP levels, no matter when the observation was made ($P = 0.03$, $P = 0.10$, $P = 0.02$,

Table 2. [^{125}I]EGF binding assays on cell lysates

Cell line and treatment	High affinity, low capacity Kd 1 (nM)	[EGFR]1 (pM)	Low affinity, high capacity Kd 2 (nM)	[EGFR]2 (pM)	n*
MDA-MB-231					
Control	0.93 ± 0.11	414.5 ± 37.4	15.08 ± 1.50	1740.2 ±234.9	5
Forskolin	0.92 ± 0.17	420.3 ±59.6	16.01 ± 0.61	1788.1 ± 180.6	4
NPM14T4/9					
Control	1.50 ± 0.15	300.1 ± 47.7	16.50 ± 1.94	1158.6 ± 235.6	4
Forskolin	1.42 ± 0.21	356.6 ± 50.6	15.31 ± 3.14	1268.5 ± 221.4	3

*Number of experiments. Cells were treated with and without 0.1 mM forskolin for 48 h. [^{125}I]EGF binding was performed on cell lysates (0.1 mg protein/sample). Increasing amounts of [^{125}I]EGF were added to the samples with and without a 200-fold excess of unlabelled EGF. Specific binding (apparent Kd and binding site concentrations) was calculated according to Scatchard.

Table 3. Intracellular cyclic AMP level of 6-h treated cells

Treatments	Cell lines			
	MDA-MB-231		NPM14T4/9	
	Mean	(S.E.)	Mean	(S.E.)
Control	15.2*	(3.2)	23.4	(5.5)
8Br-cAMP 0.1 mM	49.4	(3.1)	65.0	(14.8)
Forskolin 0.1 mM	39.1	(0.2)	122.3	(15.3)
Cholera toxin 50 nM	20.0	(0.6)	63.8	(13.6)

*10^{-6} × pmol of cAMP/cell. Cells were cultured in 24-multiwell plates and 0.1 mM 8Br-cAMP, 0.1 mM forskolin, or 50 nM cholera toxin were added to the culture medium for 6 h. Determination of their intracellular cAMP levels was performed on acetylated samples using the RIA-NEN protocol.

respectively), (ii) EGF combined to these drugs did not change them ($P = 0.82, P = 0.65, P = 0.56$, respectively).

DISCUSSION

The presence of EGFR in breast cells does not guarantee that the cells will respond to EGF [24]. EGF was found to stimulate growth of NPM14T4/9 cells but did not stimulate MDA-MB-231 cells, in standard medium. However, there was no substantial difference in the structural properties (binding capacity, affinity, size) of EGFR between MDA-MB-231 and NPM14T4/9 cell lines. Only in the presence of forskolin did EGF appeared to have a detectable stimulatory effect on MDA-MB-231 growth. This synergistic effect of forskolin was quite specific to this drug since neither cholera toxin nor 8Br-cAMP or any modifications in serum concentration changed the effect of EGF. Moreover, only in the presence of forskolin did EGF modify the cell cycle distribution of MDA-MB-231. Indeed, these cells were blocked in G1 phase specifically by forskolin. The co-treatment with EGF allowed MDA-MB-231 to overcome the cell cycle block. Thus, a G1 block seems to be necessary for an EGF effect to become visible in MDA-MB-231. None of the above effects of forskolin were observed with the EGF-sensitive NPM14T4/9 cell-line. As previously reported by Das [25], it is unlikely that a simple model involving a single signal generated by receptor–EGF interaction is sufficient to explain the complexity of the mitogenic action of EGF: it is more likely that multiple signals generated at various steps in the EGF receptor-mediated sequence of events are required for stimulation of mitosis. When MDA-MB-231 cells were treated with forskolin, no alterations in EGF binding were observed, supporting the theory that the mitogenic activity of EGF *in vitro* also depends on factors other than the level of EGFR.

Forskolin has been previously described as a direct activator of the membrane-associated adenylate cyclase [26, 27]. In contrast, activation of this adenylate cyclase by cholera toxin is indirect since it is mediated by a G-protein ADP ribosylation [28]. The increase of the intracellular cAMP, induced either by forskolin or by cholera toxin, confirms that the adenylate cyclase was functional in both cell lines. The cAMP level also increased when the cells were treated with cAMP analogues which permeate the cell membrane. Thus, whatever the level of cAMP is, under 8Br-cAMP, forskolin or cholera toxin treatment, EGF cannot change it. An accumulation of cAMP is a necessary condition for stimulation of the cAMP-dependent protein kinase (PKA); this stimulation may be an early step leading to the inhibition of growth of both MDA-MB-231 and NPM14T4/9, as described above. Although the three compounds activate the same protein kinase pathway, their effects on MDA-MB-231 growth change with a concomitant EGF treatment. Since addition of EGF does not change the inhibitory effect of 8Br-cAMP or of cholera toxin, and does not modify the cell cycle distribution, it is probable that the stimulation of MDA-MB-231 by EGF plus forskolin is not related to the intracellular level of cAMP, and that the growth rate does not depend only on cAMP. The inability of cholera toxin and 8Br-cAMP to reproduce the effect of forskolin is consistent with the existence of an additional forskolin-dependent but cAMP-independent mechanism of growth control. Indeed, recent studies have shown that forskolin can act through a mechanism which does not involve the production of cAMP in other biological models [29–33]. Moreover, in agreement with the results of Mandla and colleagues [33], we observed that concentrations of forskolin equal or higher to 0.1 mM are needed *in vitro* for a cAMP-independent effect to become manifest. Our data show, for the first time, that in breast cancer cells forskolin may also have multiple effects on the cells using cAMP-dependent and -independent mechanism.

In summary, we present evidence for a specific effect of EGF resulting in a reversal of forskolin inhibition of MDA-MB-231. This effect was not observed on the EGF-sensitive NPM14T4/9 cell line. An analogous model has been proposed for the combined action of arachidonic acid or its subsequent metabolism and EGF on fibroblastic cells transformed by oncogenic DNA viruses [34, 35]. Other studies are clearly required to elucidate the mechanism of EGF-induced mitogenesis in mammalian transformed cells.

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