

Apigenin and LY294002 prolong EGF-stimulated ERK1/2 activation in PC12 cells but are unable to induce full differentiation

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Abstract In rat pheochromocytoma cell line (PC12) cells, initial epidermal growth factor (EGF)-stimulated extracellular signal-regulated protein kinases 1/2 (ERK1/2) phosphorylation was similar to that promoted by nerve growth factor (NGF), but declined rapidly. Pre-treatment with apigenin or LY294002 sustained EGF-stimulated ERK1/2 phosphorylation whereas wortmannin partially blocked initial ERK1/2 phosphorylation. Changes in ERK1/2 phosphorylation correlated with alterations in p90 ribosomal S6 kinase activity. Wortmannin, LY294002 and apigenin totally blocked growth factor-induced protein kinase B phosphorylation. However, none of them potentiated Raf activation, which was in fact decreased by LY294002 and wortmannin. The sustained EGF-induced ERK1/2 activation promoted by apigenin was not sufficient to commit PC12 cells to differentiate, which was achieved by stimulation with NGF, either alone or in the presence of apigenin. © 2002 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.

Key words: Extracellular signal-regulated protein kinases 1/2; p90 ribosomal S6 kinase; Protein kinase B; Raf; Epidermal growth factor; Nerve growth factor

1. Introduction

Epidermal growth factor (EGF) and nerve growth factor (NGF) are potent activators of the classical extracellular signal-regulated protein kinases 1/2 (ERK1/2) (or p42/p44 MAP kinases) pathway in the rat pheochromocytoma cell line (PC12) cells [1]. However, EGF leads to stimulation of proliferation whereas NGF induces PC12 differentiation to a neurone-like phenotype. The temporality of ERK1/2 activation, very transient for EGF and sustained for several hours by NGF, seems to act as a crucial decision point for the cell commitment towards a specific fate [1]. Differential effects of EGF and NGF via Ras and Rap1 on protein kinase Raf isoforms [2], and the requirement of the phosphoinositide 3-kinase (PI3K) pathway [3] have been reported to be in-

volved in the activation of ERK1/2 evoked by NGF. Nonetheless, protein kinase B (PKB) (or Akt), one of the best known downstream transmitters of PI3K-dependent effects, has been recently shown to phosphorylate c-Raf-1 and, specially, B-Raf on inhibitory sites [4,5]. Thus, it was conceivable that activation of PKB might result in the deactivation of the Raf/mitogen-activated protein kinase (MAPK)/ERK kinase/ERK (Raf/MEK/ERK) pathway.

The fungal metabolite wortmannin, and the polyphenolic compounds LY294002 and apigenin are two types of cell-permeant pharmacological inhibitors of PI3K [6–8]. Wortmannin is effective in the nanomolar range and causes the irreversible, covalent modification of lysine-802 of the PI3K catalytic subunit [6], whereas LY294002 and apigenin compete for the ATP-binding domain in a reversible manner with IC₅₀ values in the micromolar range [7,8]. Under some circumstances, wortmannin and LY294002 have been proving to block the ERK1/2 activation pathway in some cell lines [9,10], but their effectiveness in blocking NGF induction of PC12 differentiation is more controversial [11,12]. Regarding the effects of apigenin on cell differentiation, it has been shown that this compound promoted neurite outgrowth and differentiation of B104 rat neuronal cells [13], but in contrast, interfered with the expression of myofibroblast phenotype in cultured human embryonic lung fibroblast IMR-90 [14]. Furthermore, apigenin had also been reported to inhibit ERK1/2 activation in rapid proliferating cells [15] but it seems to have little effect on IMR-90 lung fibroblasts [14]. In this work we studied the influence of wortmannin, LY294002 and apigenin on the ERK1/2 activation pathway promoted by EGF in PC12 cells, and their effects on neurite outgrowth as compared to that promoted by NGF.

2. Materials and methods

2.1. Reagents and antibodies

NGF and EGF were purchased from Life Technologies, apigenin from Sigma, wortmannin and LY294002 from Calbiochem. Protein G-Sepharose, glutathione-Sepharose, [γ -³²P]ATP and ECL were from Amersham-Pharmacia. Anti-ERK1/2 (1 μ g/ml), anti-phospho-PKB α (Ser473) (1 μ g/ml) and anti-rsk2 antibodies (2.5 μ g/200 μ g protein sample) were from Upstate Biotech. Anti-phospho-MAPK (1/2000) was from New England Biolabs. Anti-pan-Ras antibody OP40 was from Oncogene Sciences (1/100) and anti-Raf-1 antibody (2.5 μ g/200 μ g protein sample), from Transduction Laboratories. Tissue culture reagents were from Gibco.

2.2. Culture of PC12 cells and preparation of lysates

PC12 cells were cultured at 37°C in a 95/5 air/CO₂ water saturated atmosphere in 75 cm² collagen-coated flasks in Dulbecco's modified Eagle's medium (DMEM) containing 10% heat-inactivated horse se-

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Abbreviations: EGF, epidermal growth factor; ERK1/2, extracellular signal-regulated protein kinases 1/2; MAPK, mitogen-activated protein kinase; MEK, MAPK/ERK kinase; MBP, myelin basic protein; NGF, nerve growth factor; p90rsk, p90 ribosomal S6 kinase; PC12, rat pheochromocytoma cell line; PI3K, phosphoinositide 3-kinase; PKB, protein kinase B; RBD, Ras-binding domain of Raf-1

rum, 5% heat-inactivated foetal bovine serum, 2 mM L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin. For treatments, the cells were transferred to 60 mm collagen-coated dishes (3×10^6 cells per dish) or to 100 mm dishes (10^7 cells per dish). After 48 h the cells were starved in DMEM overnight and treated with NGF (50 ng/ml) or EGF (100 ng/ml). When used, inhibitors were added to the dishes 5 min before the stimulation with growth factors. Cells were harvested at the indicated times, washed with cold PBS and lysed with buffer A (50 mM Tris-HCl, pH 7.5, 0.27 M sucrose, 1 mM EGTA, 1 mM EDTA, 50 mM NaF, 1 mM sodium orthovanadate, 10 mM Na β -glycerolphosphate, 5 mM PPI, 1% Triton X-100, 1 mM benzamide, 1 mM phenylmethylsulfonylfluoride (PMSF), and 0.1% (v/v) 2-mercaptoethanol) for ERK1/2 and p90 ribosomal S6 kinase (p90rsk) assays or with REB buffer (30 mM Tris-HCl, pH 7.5, 100 mM NaCl, 0.1 mM EDTA, 1% Triton X-100, 10% glycerol, 5 mM NaF, 0.2 mM sodium orthovanadate, 0.3% (v/v) β -mercaptoethanol, 1 mM benzamide and 0.1 mM PMSF) for Ras and Raf assays. After 15 min on ice the lysate was removed and centrifuged at $13\,000 \times g$ during 15 min at 4°C. Supernatants were used for Western blotting or immunoprecipitation.

2.3. Western blot analysis

The protein content of cellular extracts was quantified by Bradford assay [16]. For Western blot experiments 50 µg of protein were loaded on SDS-PAGE gels and transferred onto PVDF membranes. The membranes were incubated with the antibodies at the indicated concentrations and developed using the enhanced chemiluminescence reagent (ECL).

2.4. Immunocomplex p90rsk activity assays

Protein G-Sepharose (5 µl bed volume) washed twice with buffer A was incubated for 30 min at 4°C with 2.5 µg of anti-p90rsk antibody and washed twice with buffer A plus 0.5 M NaCl and twice with buffer A. Then, 200 µg of cell extracts were added, and incubated for 1 h at 4°C under vigorous shaking. The beads were washed twice with buffer A plus 0.5 M NaCl, twice with buffer A and once with kinase buffer (50 mM Tris-HCl, pH 7.5, 1 mM EGTA and 2.5 mM Mg-acetate). p90rsk activity in the immunoprecipitate was measured using the synthetic peptide crosslinker (GRPTSSFAEG) at a final concentration of 30 µM and 100 µM [γ - 32 P]ATP in a final volume of 50 µl. Phosphorylation was carried out for 30 min at 30°C, and then 40 µl of the mixture was spotted on a P81 Whatman paper. The papers were washed five times with 0.5% orthophosphoric acid, once with acetone and air dried. Radioactivity was quantified in a scintillation counter.

2.5. Measurement of Ras activation

The ability of Ras-GTP to bind to RBD (Ras-binding domain of Raf-1) was used to analyse the amount of active Ras. Cells lysates (2.5 mg) were incubated with 75 µg of GST-RBD bound to glutathione-Sepharose beads for 2 h at 4°C. Beads were washed four times with REB buffer. Bound proteins were eluted by the addition of 25 µl of Laemmli loading buffer and immunoblotted with pan-Ras antibody as described above. 50 µg of cell lysates were also analysed to ensure equal quantities of Ras in the assay.

2.6. Raf immunoprecipitation and kinase assay

Cell extracts (200 µg) were incubated with 2.5 µg of anti-Raf-1 antibody prebound to 20 µl of protein G-Sepharose beads for 2 h at 4°C. The anti-Raf-coated beads were washed once with buffer B (20 mM Tris-HCl, pH 7.5, 100 mM NaCl, 2 mM EDTA, 5 mM MgCl₂, 1% Triton X-100, 5 mM NaF, 10% glycerol, 0.5% (v/v) β -mercaptoethanol, 1 mM benzamide and 0.1 mM PMSF) plus 1 M KCl, once with buffer B plus 100 mM KCl and once with buffer B. The immunoprecipitates were resuspended in 20 µl of buffer C (30 mM Tris-HCl, pH 7.5, 0.1 mM EDTA, 0.3% (v/v) β -mercaptoethanol, 10 mM MgCl₂, 0.1% Triton X-100, 5 mM NaF, 0.2 mM sodium orthovanadate) containing 0.8 mM ATP, 10 µg/ml GST-MEK and 100 µg/ml GST-ERK2 and incubated for 30 min at 30°C on a shaking platform. The reaction was stopped by addition of 20 µl of KILL buffer (30 mM Tris-HCl, pH 7.5, 6 mM EDTA, 0.3% (v/v) β -mercaptoethanol, 0.1% Triton X-100, 5 mM NaF and 0.2 mM sodium orthovanadate). The samples were centrifuged at $13\,000 \times g$ for 1 min and 6 µl aliquots of the supernatant were added to 24 µl of myelin basic protein (MBP) buffer (50 mM Tris-HCl, pH 7.5, 0.1 mM EDTA, 0.3% (v/v) β -mercaptoethanol, 10 mM MgCl₂, 0.1% Triton X-100, 5 mM NaF, 0.2

mM sodium orthovanadate and 0.1 mM [γ - 32 P]ATP (400 000 cpm/nmol), 2 mg/ml bovine serum albumin and 0.5 mg/ml MBP). After 15 min incubation at 30°C, 24 µl of the mixture was spotted on a P81 Whatman paper and the incorporation of phosphate into MBP determined as in the p90rsk assay.

2.7. Detection of cellular differentiation

Cells were plated at a density of 3×10^5 cells/60 mm dish and the next day were treated with either NGF, EGF with or without the

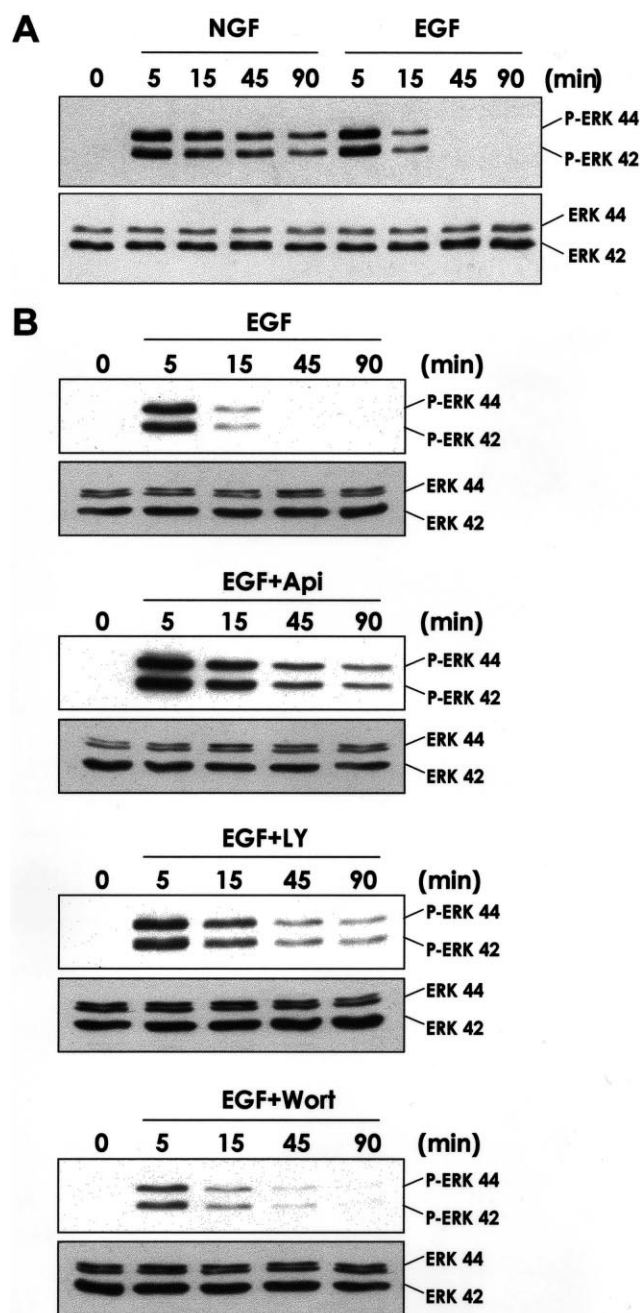


Fig. 1. Apigenin and LY294002 sustained the phosphorylation of MAP kinase activated by EGF. A: PC12 cells were serum starved overnight and then treated with 50 ng/ml NGF or 100 ng/ml EGF for the indicated times. B: Serum-starved PC12 cells were untreated or treated with either 40 µM apigenin, 50 µM LY294002 or 100 nM wortmannin for 5 min before stimulation with 100 ng/ml EGF. ERK1/2 were detected using an antibody against dual phosphorylated/activated forms of ERK (upper panel). In order to check that the same amounts of ERK were loaded, an antibody against the unphosphorylated forms was used (lower panel).

simultaneous presence of inhibitors. After 48 h of treatment, the medium was removed and the cell morphology observed in a Leica DRMB microscope and images captured with the Leica DC viewer software.

3. Results

3.1. Apigenin and LY294002 sustained ERK1/2

phosphorylation and p90rsk activation in response to EGF

Exposure of serum-starved PC12 cells to EGF or NGF caused a rapid phosphorylation of ERK1/2 (Fig. 1A). The response elicited by EGF was transient whereas NGF showed a sustained activation, which agreed with previous reports [1,2]. Pre-treatment of cells for 5 min with 100 nM wortmannin partially blocked ERK1/2 phosphorylation stimulated by EGF. In contrast, a similar pre-treatment with 40 μ M apigenin or 50 μ M LY294002 did not block EGF-induced ERK1/2 phosphorylation (Fig. 1B). Interestingly, the decline in the initial phosphorylation observed in the presence of these two inhibitors was slower than that observed with EGF alone. It is also worth noting that although the maximal phosphorylation observed in the presence of EGF plus wortmannin was lower than that caused by EGF alone, the rate of dephosphorylation was slightly slower in the presence of this PI3K inhibitor.

As apigenin has been reported to be a MAP kinase inhibitor and LY294002 is structurally related to apigenin, we wanted to check if signalling events that led to ERK1/2 phosphorylation were propagated downstream. In order to do that, we monitored the activation of p90rsk, a downstream substrate of MAP kinase. The changes detected in p90rsk activity were in good agreement with those expected from the results on ERK1/2 (Fig. 2). The deactivation in the presence of apigenin and LY294002 was slower than that observed with EGF alone reaching a plateau value between 45 and 90

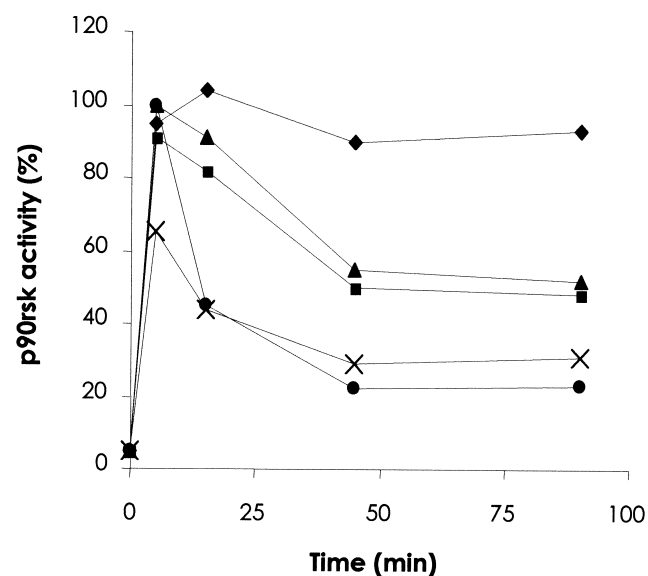


Fig. 2. Apigenin and LY294002 promoted sustained activation of p90rsk by EGF. PC12 cells were treated as in Fig. 1B and p90rsk was immunoprecipitated from 200 μ g of cell lysate. ♦ 50 ng/ml NGF, ● 100 ng/ml EGF, ▲ 40 μ M apigenin, ■ 50 μ M LY294002 and × 100 nM wortmannin. p90rsk activity was assayed using crossride as a substrate and activities are represented taking the value at 5 min after stimulation with EGF as 100%.

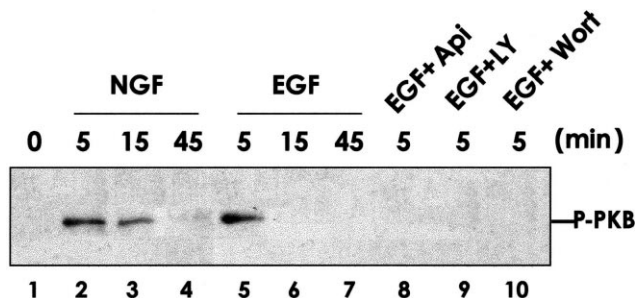


Fig. 3. Inhibition of PKB activity by PI3K inhibitors. PC12 cells were not treated (lane 1) or treated with 50 ng/ml NGF (lanes 2–4) or 100 ng/ml EGF (lanes 5–10) for the indicated times. Before the stimulation with EGF, cells were treated for 5 min with 40 μ M apigenin (lane 8), 50 μ M LY294002 (lane 9) or 100 nM wortmannin (lane 10).

min that was 50% of that attained after NGF stimulation. Wortmannin promoted a partial blockage (40%) of the initial activation and the rate of inactivation was similar to the one observed with EGF.

3.2. PI3K inhibitors block PKB activation in response to EGF or NGF

The different effects on ERK1/2 activation caused by wortmannin and apigenin or LY294002 might reflect differences in the cellular uptake of these compounds, which could interfere with their ability to block the PI3K pathway. To explore this possibility, the effect of these three inhibitors on the phos-

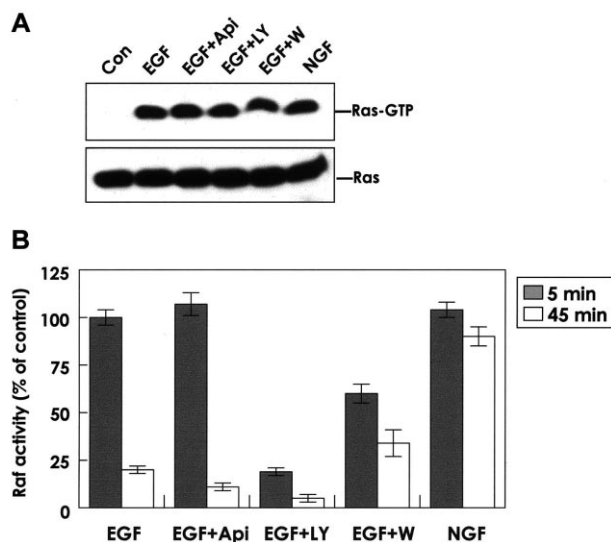


Fig. 4. Effect of PI3K inhibitors on Ras and Raf activity. A: Serum-starved PC12 cells were untreated (Con) or treated for 2 min with EGF (100 ng/ml), EGF plus either 40 μ M apigenin, 50 μ M LY294002 or 100 nM wortmannin, or with NGF (50 ng/ml). Cells were lysed and Ras-GTP was determined by precipitating with GST-RBD bound to glutathione-Sepharose, followed by electrophoresis in 12% SDS-PAGE gels and Western blotting, as indicated in Section 2 (upper panel). Equal quantities of cell extracts were analysed by Western blot to ensure equal amounts of total Ras in the assay (lower panel). B: Raf activity was immunoprecipitated from cell extracts treated at the indicated times with EGF (100 ng/ml), EGF plus either 40 μ M apigenin, 50 μ M LY294002 or 100 nM wortmannin, or with NGF (50 ng/ml) and assayed in a coupled kinase assay which measures the phosphorylation of MBP. Activities are represented taking the value at 5 min after stimulation with EGF as 100%.

phorylation of PKB promoted by EGF and NGF was monitored. Exposure of PC12 cells to either EGF or NGF promoted a rapid increase in phospho-PKB, being the response to NGF more persistent than that to EGF (Fig. 3). The simultaneous addition of wortmannin, apigenin or LY294002 totally blocked the appearance of phospho-PKB in response to either EGF or NGF. This confirmed that the inhibitors had reached PI3K inside the cell.

3.3. PI3K inhibitors do not affect Ras and have different effects on Raf activation

The possibility that the sustained activation of ERK1/2 in response to apigenin and LY290042 may be due to effects of these compounds on Ras or Raf was tested. No significant effects of the inhibitors were observed on EGF-induced Ras stimulation (Fig. 4A). Raf activation attained in response to EGF, EGF plus wortmannin or NGF was in good agreement with the activation kinetics of ERK1/2 and p90rsk (Fig. 4B). In contrast, neither apigenin nor LY294002 sustained EGF-stimulated Raf activity. In fact, the presence of LY294002 resulted in a very low Raf activity at 5 min after EGF stim-

ulation. This was a surprising result in view of the sustained activation of ERK1/2 caused by these two compounds.

3.4. The initially sustained activation of ERK1/2 is not sufficient to commit PC12 cells to complete differentiation

The consequences of the partially sustained ERK1/2 pathway activation achieved by EGF plus apigenin on neurite outgrowth were then studied. In agreement with previous reports, stimulation with NGF altered cell morphology, inducing in many cells the development of filamentous forms 90 min after NGF addition (Fig. 5, central panels, left) whereas the unstimulated cells showed a rounded morphology (Fig. 5, upper panels, left). Some cells displaying protrusions and short filaments were also observed after EGF treatment (Fig. 5, upper panels, right), a fact that was more marked when the cells were stimulated with EGF plus apigenin (Fig. 5, central panels, right). When cell cultures were allowed to proceed for 48 h, the NGF-treated cells acquired a morphology typical of differentiated PC12 cells (Fig. 5, lower panels, left), whereas control cells did not differentiate (not shown). Both the cells treated with EGF alone or plus apigenin showed some short protrusions resembling collapsed initial neurites, but the differentiation process did not proceed any further (not shown). The possibility that the lack of differentiation was due to toxic effects of apigenin is unlikely since its presence did not affect PC12 differentiation induced by NGF (Fig. 5, lower panels, right).

4. Discussion

The mechanisms underlying the different temporality of ERK1/2 activation in response to NGF and EGF are important questions that have been addressed by different groups by experimental approaches [1–3] and mathematical models [17], with controversial conclusions. In recent years it has become evident that the PI3K pathway may affect ERK1/2 activation not only positively through Ras/c-Raf-1 [1,2], Rap/B-Raf [2,3] or by Cdc42/Rac→Pak→c-Raf-1 [10], but also negatively through inactivating phosphorylations of c-Raf and/or B-Raf by PKB [4,5]. The results obtained in our present work with wortmannin would agree with the hypothesis that PI3K activity is important for maximal initial ERK1/2 activation in response to EGF. The lack of inhibition observed on PC12 cells with the PI3K inhibitors LY294002 and apigenin is then intriguing.

MEK phosphorylation in EGF- or NGF-stimulated PC12 cells has been estimated to be catalysed by B-Raf and c-Raf [2]. A remarkable effect shared by wortmannin, LY294002 and apigenin was the complete blockage of PKB phosphorylation. This fact may underlay their sustained ERK1/2 activation in response to EGF since it would favour the maintenance of c-Raf and B-Raf in an activated state. However, our data indicate that in this cell line blockage of PKB phosphorylation by these compounds does not result in an increase of Raf activity. Furthermore, the effect of LY294002 and apigenin on EGF-induced Raf activation was markedly different in spite of their similar effects on ERK1/2 activities. The fact that Ras activation does not change in the presence of the three inhibitors tested suggests that the effects on Raf are not mediated solely by Ras. This also suggests that apigenin, LY294002 and wortmannin might affect different sets of cellular targets. Wortmannin effects on ERK1/2 activation may

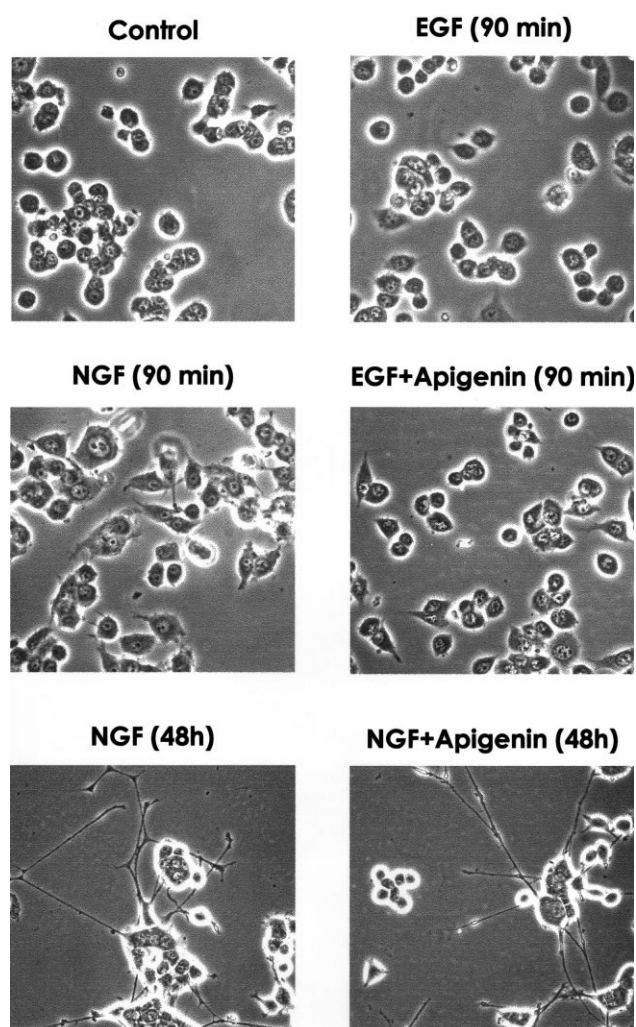


Fig. 5. Effect of apigenin on PC12 cell differentiation. PC12 were seeded in 60 mm collagen-coated dishes and 24 h later were stimulated with NGF (50 ng/ml) or EGF (100 ng/ml). Where indicated, 40 μ M apigenin was added at the same time as the growth factors.

be explained through changes in Raf activity whereas those of apigenin and LY294002 would require other undefined components which may influence ERK1/2 phosphorylation/dephosphorylation processes. A lack of correlation between Raf activity and ERK1/2 phosphorylation has been reported previously by others [18]. In any case, the data clearly indicate that in this cell line apigenin does not act as an inhibitor of ERK1/2 since a sustained activation of p90rsk, a downstream target of ERK1/2 was observed in its presence and it did not block the differentiation induced by NGF.

Maintenance of ERK1/2 activation for periods longer than those achieved with EGF alone induced mid-term morphological alterations of PC12 similar to those caused by NGF. However this was not sufficient to prime the cells to complete the differentiation process. Interestingly, overexpression of RapN17, a dominant negative mutant of Rap1, that did not affect the initial rapid activation of ERK1/2 in response to NGF but abolished the maintenance of ERK1/2 in the activated state, did not inhibit neurite outgrowth [19]. Thus, it has been suggested that NGF-induced differentiation of PC12 cells might involve additional events that occur at later stages and are triggered through PI3K-dependent pathways [11,19]. In any case, the fact that apigenin did not block NGF-induced differentiation under conditions that prevent PKB phosphorylation argues against a prominent role of the PKB pathway in triggering PC12 differentiation.

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