Bombesin and epidermal growth factor stimulate the mitogen-activated protein kinase through different pathways in Swiss 3T3 cells

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Both bombesin and epidermal growth factor (EGF) are potent mitogens in Swiss 3T3 cells that nonetheless have dissimilar receptor structures. To explore possible common intracellular events involved in the stimulation of cellular growth by these two peptides, we have evaluated the regulation of the mitogen-activated protein (MAP) kinase. Exposure of Swiss 3T3 cells to bombesin, EGF or the protein kinase C activator phorbol 12-myristate 13-acetate (PMA) causes the rapid and transient stimulation of the enzyme activity. Pretreatment of cells with the protein kinase inhibitor H-7, or down-regulation of cellular protein kinase C by prolonged exposure to PMA, causes a decrease of over 90% in the activation of MAP kinase by bombesin. In contrast, these treatments have no effect on the stimulation of MAP kinase by EGF. The stimulation of MAP kinase activity by bombesin is dose-dependent, occurring over a narrow concentration range of the peptide. Both EGF and bombesin stimulate the phosphorylation of an immunoprecipitable MAP kinase protein migrating at 42 kDa on SDS/PAGE. Phosphoamino acid analysis of this phosphorylated protein reveals that EGF and bombesin stimulate phosphorylation on tyrosine, threonine and serine residues. Tyrosine phosphorylation of the enzyme, as evaluated by anti-phosphotyrosine blotting of the immunoprecipitated protein, reveals that the time course of phosphorylation by both mitogens correlates with stimulation of enzyme activity. These results provide further evidence for the convergence of discrete pathways emanating from tyrosine kinase and G-protein-linked receptors in the regulation of MAP kinase.

INTRODUCTION

An increasing body of evidence suggests that phosphorylation of cellular proteins plays an important role in the actions of hormones, growth factors and other mitogenic stimuli. Numerous serine/threonine-specific protein kinases have been identified that may catalyse these phosphorylation reactions. One of the most promising candidates discovered thus far is the family of kinases known as the mitogen-activated protein (MAP) kinases. Although the precise molecular mechanisms involved in the activation of MAP kinase remain unclear, previous studies suggest an important role in the actions of numerous mitogens, including insulin [1], epidermal growth factor (EGF) [2,3], phorbol esters [2], nerve growth factor [4–7], platelet-derived growth factor [8], fibroblast growth factor [6], growth hormone [9], secretagogues [10], insulin-like growth factor-1 [11], interleukin-1 [12], *N*-methyl-D-aspartate [13], and interaction of T-cell surface receptors with presented antigens [14]. Additionally, extracellular signals that are thought to be mediated through G-protein-coupled receptors may also stimulate this kinase [15]. Evaluation of the substrate specificity of MAP kinase *in vitro* revealed that the enzyme can phosphorylate microtubule-associated protein-2 (MAP-2) [16], myelin basic protein [17–19], ribosomal S6 kinase II [1], the EGF receptor [20], the c-*jun* proto-oncogene [21] and *raf*-1 kinase [22,23], whereas other common substrates are not efficiently phosphorylated [24,25]. Although the function of MAP kinase *in vivo* has yet to be clarified, studies on the phosphorylation and activation of S6 kinase suggest that MAP kinase may play an important role as an integration switch in a phosphorylation cascade, perhaps involved in the regulation of protein synthesis. Moreover, the phosphorylation of c-*jun*, a component of the AP-1 transcription factor, resulted in increased activity, suggesting a potential role in stimulation of cell growth [21].

Numerous studies indicate that MAP kinase requires phosphorylation on both threonine and tyrosine residues for full activation [4,19,26,27], and that the enzyme may be phosphorylated on threonine, serine or tyrosine in response to growth factors [12,25,28,29]. Although the order of phosphorylation of these residues is unknown, the tyrosine phosphorylation of the enzyme may be a result of autophosphorylation [30] occurring secondarily to phosphorylation on threonine, which itself may result from a hormone-induced activator [31]. Alternatively, differential phosphorylation of tyrosine and/or threonine may be catalysed by exogenous kinases resulting from different pathways of activation, or by a single threonine- and tyrosine-specific protein kinase [26,31,32]. We report here that in Swiss 3T3 cells both EGF and bombesin activate MAP kinase over a similar time course, but via distinct biochemical pathways.

MATERIALS AND METHODS

Materials

All reagents were purchased from Sigma, except for tissue culture reagents (GIBCO), EGF (Collaborative Biomedical Products, Bedford, MA, U.S.A.), [γ-^{32}P]ATP (300 Ci/mmol; 1 Ci = 37 GBq) (Amersham), ^{125}I-labelled sheep anti-mouse Ig (Amersham), phorbol 12-myristate 13-acetate (PMA) (GIBCO), mouse anti-phosphotyrosine monoclonal antibody (UBI), Pansorbin (Calbiochem), Protein G plus beads (Oncogen), mouse

Abbreviations used: EGF, epidermal growth factor; MAP, mitogen-activated protein; MAP-2, microtubule-associated protein; PMA, phorbol 12-myristate 13-acetate.

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anti-(MAP kinase) monoclonal antibody (Zymed) and X-ray film (Kodak).

Cell culture

Swiss 3T3 cells were grown in Dulbecco's modified Eagle's medium containing 4500 mg of D-glucose/l and 10% fetal-bovine serum. Before hormonal treatment, the medium was replaced with serum-free medium and incubated overnight. Unless otherwise indicated, bombesin and EGF were directly added to the medium to a final concentration of 100 ng/ml and the incubation was continued for the indicated time at 37 °C. After hormonal treatment, the medium was removed, and the cell layer was quickly washed with 3 × 10 ml of ice-cold Ca^{2+}-free phosphate-buffered saline. Cells were collected with a rubber policeman and lysed as described previously [5,33].

MAP kinase assay

This was assayed as described previously, with slight modifications [5,33]. Briefly, 10 μl samples of cell lysate were incubated with MAP-2 at 0.2 mg/ml for 10 min at 30 °C in a final volume of 25 μl containing 50 mM Tris/HCl (pH 7.4), 2 mM EGTA, 10 mM $MgCl_2$ and 40 μM [γ-^{32}P]ATP (1 μCi). The reaction was stopped by the addition of 4 × Laemmli SDS/PAGE sample buffer [34], and phosphorylated MAP-2 was resolved by SDS/PAGE (7.2%-acrylamide gel). Coomassie Blue-stained bands containing phospho-MAP-2 were excised from the gels, and incorporated radioactivity was measured by Čerenkov counting. For chromatography of the enzyme, lysate prepared from one 150 mm-diam. tissue-culture dish was chromatographed on a Mono Q HR 5/5 column [5]. Fractions were immediately assayed for MAP kinase activity.

Immunoprecipitation and anti-phosphotyrosine blotting

Swiss 3T3 cells were grown in 100 mm tissue-culture dishes as described above. After hormonal treatment, cells were washed and lysed in 1% SDS containing 25 mM Tris/HCl (pH 7.4), 2 mM Na_3VO_4 and 0.2 mM Na_2MoO_4. The lysates were heated at 100 °C for 5 min, and diluted with 1 ml of RIPA buffer containing 2 mM Na_3VO_4 and 0.2 mM Na_2MoO_4. Diluted lysates were pre-cleared with 60 μl each of Pansorbin and rabbit IgG beads, and centrifuged at 14000 rev./min for 10 min to remove undissolved materials. Supernatants were aspirated and 5 μg of monoclonal mouse anti-MAP kinase antibody was added. After a 45 min incubation on ice, the immunocomplexes were precipitated with Protein G beads. The precipitated immune complexes were then subjected to SDS/PAGE and transferred to Immobilon membranes for immunoblotting with anti-phosphotyrosine antibody as described in [35].

Phosphoamino acid analysis

Dishes (60 mm diam.) of Swiss 3T3 cells were serum-starved overnight and labelled with 2 mCi of [^{32}P]P_i in 2 ml of phosphate-free Dulbecco's modified Eagle's medium for 2 h. Cells were then treated with 100 ng of bombesin or EGF/ml, and lysed with 1% SDS. MAP kinase in the cell lysate was immunoprecipitated and subjected to SDS/PAGE followed by transfer to Immobilon membrane as described above. Phosphorylated MAP kinase was detected by autoradiography and excised from the membrane. Excised MAP kinase was then hydrolysed with 6 M HCl, followed by phosphoamino acid analysis as described in [36].

RESULTS

Bombesin is a potent mitogen for Swiss 3T3 cells. Although several studies have demonstrated bombesin-induced increases in cellular tyrosine phosphorylation [37–41], cloning of the cDNA encoding the bombesin receptor predicted a protein that is a member of the rhodopsin family of receptors [42], containing seven transmembrane domains and coupled to a G-protein. To determine whether the mitogenic properties of bombesin are related to activation of MAP kinase, Swiss 3T3 cells were treated with bombesin or EGF in the presence of the protein kinase inhibitor H-7 (Figure 1). Treatment of cells with bombesin caused a 2.5-fold increase in MAP kinase activity assayed in lysates. Lysates derived from EGF-treated cells exhibited a 5-fold increase in activity. Addition to cells of the protein kinase C inhibitor H-7 blocked the stimulation observed with bombesin, but had no effect on that induced by EGF.

To characterize further the effect of bombesin, Swiss 3T3 cells were treated with bombesin or PMA. The time courses of activation of MAP kinase by EGF, bombesin and PMA were nearly identical (Figure 2). All three agents rapidly stimulated the enzyme activity, reaching a maximum by 2 min for bombesin and PMA, or 5 min for EGF, and declining thereafter. The dose-dependence of stimulation of Swiss 3T3-cell MAP kinase by bombesin was also evaluated (Figure 3). Bombesin stimulated the enzyme over a narrow concentration range, with a half-maximal concentration for activation (EC_{50}) of approx. 2 nM, and a maximal effect occurring at approx. 6 nM.

To explore further the role of protein kinase C in the bombesin-dependent activation of MAP kinase, we compared enzyme activities stimulated by bombesin or EGF in cells subjected to prolonged incubation with PMA (Table 1). Incubation of cells

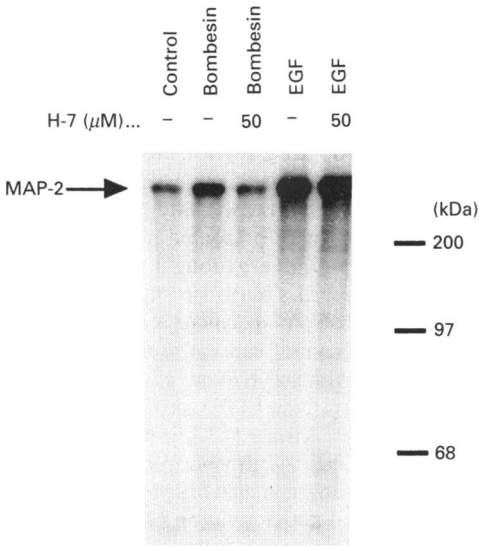

Figure 1 EGF and bombesin stimulate MAP kinase activity in Swiss 3T3 cells

Swiss 3T3 cells were treated with 100 ng of bombesin or EGF/ml for 5 min in the presence of the protein kinase inhibitor H-7 (50 μM). Lysates were incubated with [^{32}P]ATP in the presence of MAP-2 as described in the Materials and methods section. Reactions were stopped with Laemmli sample buffer and subjected to SDS/PAGE on 7.2%-acrylamide gels, followed by autoradiography. Results were repeated in four separate experiments.

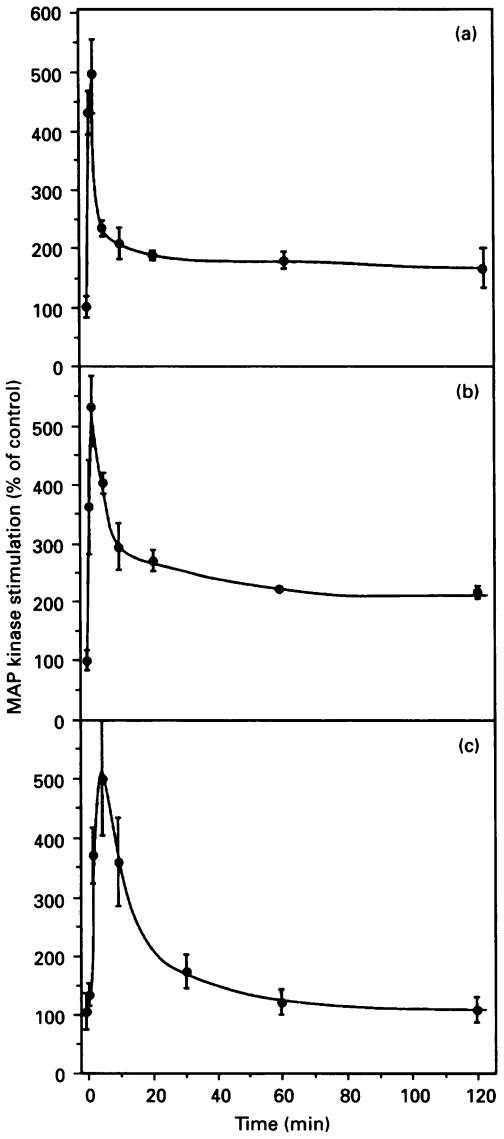

Figure 2 Time course of the activation of MAP kinase by EGF, bombesin or PMA

Overnight-serum-starved Swiss 3T3 cells were treated with 100 ng of bombesin/ml (**a**), 100 nM PMA (**b**) or 100 ng of EGF/ml (**c**) for the indicated time. Cells were lysed and MAP kinase activity in cell lysates was assayed as described in Figure 1. Results are expressed as means ± S.D. of triplicate determinations and were repeated three times.

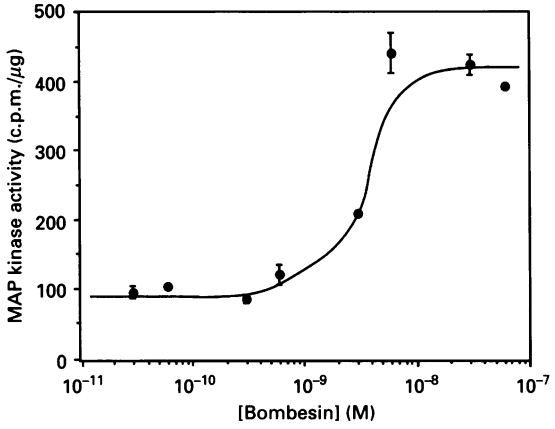

Figure 3 Dose-dependence of MAP kinase activities by bombesin

Overnight-serum-starved Swiss 3T3 cells were treated with the indicated concentrations of bombesin for 5 min. Cells were then lysed and MAP kinase activity in cell lysates was assayed as described in Figure 1. Results are expressed as means ± S.D. of triplicate determinations and were repeated three times.

Table 1 Prolonged incubation with phorbol ester attenuates stimulation of MAP kinase by bombesin but not by EGF

Swiss 3T3 cells were treated with or without PMA (100 nM) in serum-free Dulbecco's medium overnight, and then with bombesin (100 ng/ml), EGF (100 ng/ml) or PMA (100 nM) for 5 min. Cells were lysed and MAP kinase activity in cell lysates was assayed as described in Figure 1. Results are expressed as means ± S.D. of triplicate determinations and were repeated three times.

	MAP kinase activity (c.p.m./μg) from cells treated with:			
Preincubation	Untreated	EGF (100 ng/ml)	Bombesin (100 ng/ml)	PMA (100 nM)
None	21.9 ± 2.6	157.7 ± 27.5	53.8 ± 5.7	92.6 ± 2.4
PMA (overnight)	26.8 ± 2.1	161.4 ± 10.2	25.4 ± 2.8	23.9 ± 2.2

with 100 nM PMA caused almost total down-regulation of protein kinase C activity (result not shown), and desensitized the enzyme to further activation by the tumour promoter. Prolonged incubation with PMA caused over a 90% decrease in the activation of MAP kinase by bombesin. In contrast, protein kinase C down-regulation did not compromise the stimulation of MAP kinase by EGF. These results suggest that the full stimulation of MAP kinase by bombesin, but not by EGF, requires functional protein kinase C in Swiss 3T3 cells.

MAP kinase activities in lysates from bombesin- or EGF-treated Swiss 3T3 cells were chromatographed on a f.p.l.c. ion-exchange column (results not shown). Both growth factors produced identical chromatographic profiles for MAP kinase on Mono Q anion-exchange chromatography. The nearly identical profiles of MAP-2 phosphorylation indicated that bombesin and EGF activated the same molecular species of MAP kinase in Swiss 3T3 cells.

To explore further the differences in activation of MAP kinase by bombesin and EGF, we evaluated the mitogen-dependent phosphorylation of the enzyme. Swiss 3T3 cells were preincubated with [^{32}P]P_i, followed by a 5 min treatment with bombesin or EGF. After hormone treatment, lysates were prepared and immunoprecipitated with anti-(MAP kinase) antibody, followed by electrophoresis on 7.2%-polyacrylamide gels and autoradiography (Figure 4). Both mitogens stimulated the incorporation of ^{32}P into an immunoprecipitated protein migrating at 42 kDa, exactly at the position of the pp42 MAP kinase, or ERK-2 [43,44], as detected by immunoblotting with this antibody (results not shown). PMA also produced a similar incorporation of ^{32}P into this band (results not shown). The ^{32}P-labelled immunoprecipitated bands were excised from the gel and subjected to phosphoamino acid analysis (Figure 4b). Although EGF and bombesin both caused the phosphorylation of tyrosine, threonine and serine residues on the protein, the proportions of phosphorylation on the three amino acid residues were different. In cells treated with EGF, phosphotyrosine was the predominant phosphoamino acid, whereas bombesin treatment caused a greater increase in serine phosphorylation. The detection of

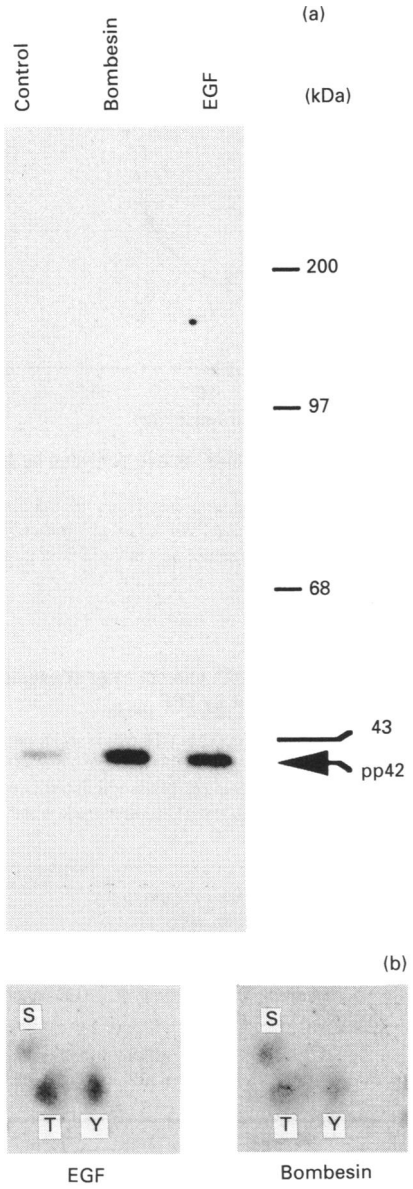

Figure 4 MAP kinase is phosphorylated on tyrosine and serine in response to bombesin

(**a**) Plates of Swiss 3T3 cells were preincubated with [^{32}P]P_i, followed by a 5 min treatment with 100 ng of bombesin or EGF/ml. After hormonal treatment, lysates were prepared and immunoprecipitated with anti-(MAP kinase) antibody, followed by electrophoresis on 7.2%-polyacrylamide gels and autoradiography. (**b**) The ^{32}P-labelled immunoprecipitated bands migrating at 42 kDa (pp42) were excised from the gel and subjected to phosphoamino acid analysis. Results are representative of a single experiment that was repeated five times.

phosphoserine, phosphothreonine and phosphotyrosine residues in response to EGF and bombesin is similar to that observed with both pp42 and pp44 MAP kinase in KB epidermoid carcinoma cells treated with interleukin-1 [12] or murine EL4 cells treated with phorbol 12,13-dibutyrate [28]. In both of these cases, the mitogens caused phosphate incorporation into serine, threonine and tyrosine residues. Results presented here are different from those observed in *Xenopus* M-phase-activated MAP kinase [25] and sea-star p44^{mapk} [45], in which only phosphoserine and phosphotyrosine were detected. Moreover, treatment of Chinese-hamster ovary cells with insulin or phorbol ester caused increases in phosphothreonine and phosphotyrosine, although phosphoserine was not detected [29].

The kinetics of tyrosine phosphorylation of the enzyme were evaluated by Western blotting of anti-MAP kinase immunoprecipitates with anti-phosphotyrosine antibody (Figure 5). Bombesin caused the rapid tyrosine phosphorylation of pp42^{mapk}. The phosphoprotein was observed within 2 min of exposure of cells to the mitogen, and disappeared after 5 min, consistent with the time course of stimulation of MAP kinase activity by this agent. In contrast, tyrosine phosphorylation of the 42 kDa protein observed in response to EGF persisted for up to 10 min, and then declined at 30 min, also consistent with the time course of the stimulation of MAP kinase activity by EGF, as shown in Figure 2.

DISCUSSION

Considerable evidence now indicates that the activation of MAP kinase is a common event in the control of cellular growth [46]. This enzyme is activated in a variety of cell types, and in response to numerous growth factors whose receptors are structurally unrelated, suggesting the existence of distinct pathways that converge at this site of regulation. An example of two such converging signalling pathways lies in the regulation of Swiss 3T3 cells by EGF and bombesin. Both agents are potent mitogens that nonetheless have dissimilar receptor structures. The EGF receptor is itself a tyrosine kinase with a single transmembrane domain [47], whereas the bombesin receptor belongs to the rhodopsin superfamily, containing seven transmembrane domains [42], coupled to a G-protein, and subsequently linked to polyphosphoinositide turnover, intracellular Ca^{2+} mobilization and protein kinase C activation [48,49]. To explore the similarities and differences in the cellular actions of these two mitogens, we have compared the pathways leading to activation of MAP kinase. We demonstrate here that, although both peptides cause the rapid stimulation of identical molecular species of the enzyme, differences exist in the modes of activation. The protein kinase inhibitor H-7, as well as down-regulation of protein kinase C by prolonged incubation of cells with phorbol ester, completely blocked activation of the enzyme by bombesin, but did not attenuate the effect of EGF. Moreover, exogenous addition of phorbol ester produced kinetics of activation and inactivation of MAP kinase identical with those observed with bombesin, whereas the effect of EGF was significantly longer lived. These results are in agreement with previous observations that prolonged exposure of Swiss 3T3 cells to phorbol ester abolish the effects of bombesin on c-*fos* expression, S6 kinase activation and DNA synthesis [49]. These data, in addition to subtle differences in the phosphoamino acid patterns detected in the immunoprecipitated 42 kDa MAP kinase protein, indicate that the effect of bombesin is mediated, at least in part, through protein kinase C, whereas the EGF response is protein kinase C-independent.

Although the precise biochemical events involved in MAP kinase activation remain uncertain, data presented here support previous assertions [4,19,26,27,50] that tyrosine and threonine phosphorylations of the enzyme are critical in the activation process. As described previously [41], bombesin can stimulate tyrosine phosphorylation in 3T3 cells, although its receptor is not a tyrosine kinase. One possibility is that increased tyrosine phosphorylation of MAP kinase observed in response to bombesin or phorbol ester [51] is a result of the autophosphorylation of the enzyme on tyrosine residues. Indeed, a recent report [30] indicates that bacterially expressed p42^{mapk} can autophosphorylate on tyrosine via an intramolecular mechanism.

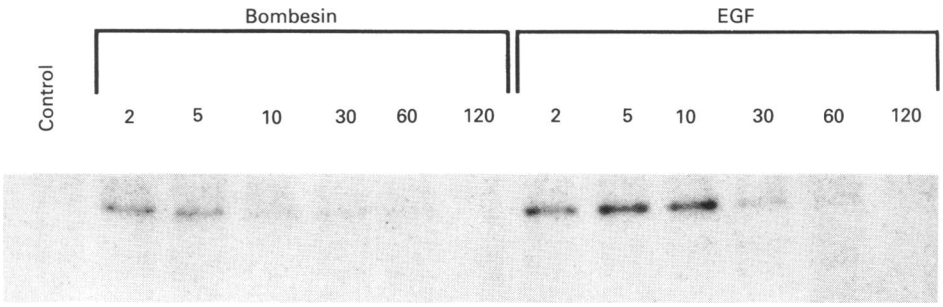

Figure 5 Time course of tyrosine phosphorylation of MAP kinase by bombesin and EGF

Swiss 3T3 cells were grown in 60 mm dishes till confluent. Before hormonal treatment, cells were serum-starved for 12 h. Each dish of cells was treated with 100 ng of bombesin or EGF/ml for the indicated times (min). Cells were then harvested and lysed in 100 μl of 1% SDS. Cell lysates were diluted with 1 ml of RIPA buffer and centrifuged. The supernatant was precipitated with 5 μg of mouse anti-(MAP kinase) antibody. Immunoprecipitated MAP kinase was then subjected to SDS/PAGE, followed by Western blotting with anti-phosphotyrosine antibody. Results are representative of a single experiment that was repeated four times.

Alternatively, activation of protein kinase C may lead to the phosphorylation and subsequent activation of one (or more) secondary kinase(s) that may itself phosphorylate MAP kinase on serine, threonine and/or tyrosine, causing activation. Further investigation into the pathways of activation may reveal the mechanisms by which MAP kinase can integrate mitogenic stimuli from different membrane signals.

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