Activation of Extracellular-Signal Regulated Kinase by Epidermal Growth Factor Is Potentiated by cAMP-Elevating Agents in Primary Cultures of Adult Rat Hepatocytes

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We investigated the effects of α- and β-adrenergic agonists on epidermal growth factor (EGF)-stimulated extracellular-signal regulated kinase (ERK) isoforms in primary cultures of adult rat hepatocytes. Hepatocytes were isolated and cultured with EGF (20 ng/ml) and/or α_1-, α_2- and β_2-adrenergic agonists. Phosphorylated ERK isoforms (ERK1; p44 mitogen-activated protein kinase (MAPK) and ERK2; p42 MAPK) were detected by Western blotting analysis using anti-phospho-ERK1/2 antibody. The results show that EGF induced a 2.5-fold increase in ERK2-, but not ERK1-, phosphorylation within 3 min. This EGF-induced ERK2 activation was abolished by treatment with the EGF-receptor kinase inhibitor AG1478 (10^{-7} M) or the MEK (MAPK kinase) inhibitor PD98059 (10^{-6} M). The α_2-adrenergic and β_2-adrenergic agonists, UK14304 (10^{-6} M) and metaproterenol (10^{-6} M), respectively, had no effect in the absence of EGF, but metaproterenol significantly potentiated EGF-induced ERK2 phosphorylation. Moreover, the cell-permeable cAMP analog 8-bromo cAMP (10^{-7} M), also potentiated EGF-induced ERK2 phosphorylation. The effects of these analogs were antagonized by the protein kinase A (PKA) inhibitor H-89 (10^{-7} M). These results suggest that direct or indirect activation of PKA represents a positive regulatory mechanism for EGF stimulation of ERK2 induction.

Key words epidermal growth factor; extracellular-signal regulated kinase; hepatocyte; α_2-adrenergic agonist; β_2-adrenergic agonist; cross-talk

Epidermal growth factor (EGF) is well recognized as a potent mitogen, and appears to trigger liver regeneration after partial hepatectomy or after acute liver cell necrosis caused by chemicals *in vivo*.[1—4] The response of adult rat hepatocytes to EGF has also been extensively investigated with respect to DNA synthesis and proliferation *in vitro*. EGF is now known to be a potent hepatocyte mitogen and induces multiple biological activities in a wide variety of cell types.[5—8] More recently, we reported that EGF rapidly stimulates hepatocyte DNA synthesis and proliferation during short-term (*e.g.*, 4 h) culture.[9] In addition, the hepatocyte DNA synthesis and proliferation induced by EGF was inhibited depending on the initial plating density. Furthermore, we found that EGF-induced hepatocyte DNA synthesis and proliferation were potentiated by β_2-adrenergic agonists.[9]

The signal transduction pathways activated in response to EGF in hepatocytes and other cell types are now more clearly understood.[10—12] Using specific inhibitors of signal transducers, we pharmacologically demonstrated that EGF-receptor tyrosine kinase and ribosomal p70 S6 kinase activities, but not phosphoinositide 3-kinase (PI3K), are essential for EGF-induced DNA synthesis and proliferation in primary cultures of adult rat hepatocytes.[9] PI3K is considered to be involved in hepatocyte growth factor (HGF) signal transduction.[13]

In addition, extracellular-signal regulated kinase (ERK)1/2, also known as mitogen-activated protein kinase (p42/44 MAPK), is now known to be activated in response to a large number of mitogenic stimuli, and this enzyme is a key participant in the response to various growth factors and cytokines.[14,15] In order to better understand the EGF-mediated signaling pathway, we investigated whether activation of the ERK isoforms, ERK1 and ERK2, is involved in EGF-induced DNA synthesis and proliferation in primary cultures of adult rat hepatocytes.

Catecholamines (*e.g.*, norepinephrine and its analogs) have been shown to be involved in the regulation of liver function (*e.g.*, lipid metabolism, carbohydrate metabolism and cell growth). There are several types of catecholamine receptors, β_1- and β_2-receptors, that stimulate adenylate cyclase (AC), while α_2-receptor inhibits its phosphorylation.[5,16] α_1-Receptor is involved in phospholipase C activation and subsequent increases in inositolphosphate turnover and diacylglycerol production.[17,18] However, some investigators have reported that α_1- and β-adrenergic responses are involved in adrenergic regulation of carbohydrate metabolism in the liver of normal adult rats and in cultured hepatocytes.[19,20] There are few studies regarding the adrenergic regulation of ERK1/2 phosphorylation induced by growth factors in liver cells. Therefore, in the present study, we examined whether α_1-, α_2- and β_2-adrenergic agonists can modulate EGF-induced ERK1/2 isoform activities. The physiological significance of cross-talk between the EGF pathway and α_1-, α_2- and β_2-adrenergic receptor-mediated pathways in regulating hepatocyte proliferation is also discussed.

MATERIALS AND METHODS

Materials The following reagents were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.): dexamethasone, aprotinin, EGF (human recombinant), 2,4-dideoxyadenosine, AG1478 (*N*-[3-chlorophenyl]-6,7-dimethoxy-4-quinazolinamine), 8-bromo-cAMP, LY294002 (2-(4-morpholinyl)-8-phenyl-4*H*-1-benzopyran-4-one hydrochloride), H-89 (*N*-[2-(*p*-bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide dihydrochloride), aphidicolin, metaproterenol hemisulfate, GF109203X hydrochloride (2-[1-(3-dimethylaminopropyl)-1*H*-indol-3-yl]-3-(1*H*-indol-3-yl)maleimide hydrochloride, phenylephrine hydrochloride, UK14304 (5-bromo-*N*-(4,5-dihydro-1*H*-imidazol-2-yl)-6-quinoxalinamine), PD98059 (2′-amino-3′-methoxyflavone). U-73122

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(1-[6-[[(17β)-3-methoxyestra-1,3,5(10)-trien-17-yl]amin]ohexyl]-1H-pyrrole-2,5-dione) and U-73343 (1-[6-((17β-3-methoxyestra-1,3,5(10)-trien-17-yl)amino)hexyl]-2,5-pyrrolidinedione) were obtained from Enzo Life Sciences (Farmingdale, NY, U.S.A.). Rapamycin and 12-*O*-tetradecanoylphorbol-13-acetate (phorbol ester, TPA) were obtained from Research Biochemicals Inc. (Natick, MA, U.S.A.). Williams' medium E and newborn calf serum were purchased from Flow Laboratories (Irvine, Scotland). Collagenase (type II) was obtained from Worthington Biochemical Co. (Freehold, NJ, U.S.A.). Anti-phospho-ERK1/2 monoclonal antibody, anti-ERK1/2 monoclonal antibody, anti-phospho-EGF receptor (Tyr1068) polyclonal antibody, and anti-EGF receptor antibody were obtained from Cell Signaling Technology (Danvers, MA, U.S.A.). [Methyl-^{3}H]thymidine (20 Ci/mmol) was purchased from DuPont-New England Nuclear (Boston, MA, U.S.A.). All other reagents were of analytical grade.

Animals Male Wistar rats (weight, 200—220 g) were obtained from Saitama Experimental Animal Co. (Saitama, Japan). The rats were adapted to a light-, humidity- and temperature-controlled room over a minimum 3-day period prior to the start of experiments. Rats were fed a standard diet and given tap water *ad libitum*. Animals used in this study were handled in accordance with the "Guiding Principles for the Care and Use of Laboratory Animals," as approved by the Ministry of Education, Culture, Sports, Science and Technology of Japan and the Guidelines for the Care and Use of Laboratory Animals of Josai University.

Hepatocyte Isolation and Culture Male Wistar rats were anesthetized by intraperitoneal injection of sodium pentobarbital (45 mg/kg). Two-step *in situ* collagenase perfusion was performed to facilitate disaggregation of adult rat livers, as described previously.[21] After perfusion, cells were dispersed in Ca^{2+}-free Hanks' solution. Cells were then washed three times by slow centrifugation (120 $g\times$1 min). Hepatocyte viability was monitored using Trypan blue dye exclusion. On average, more than 96% of the cells remained in-

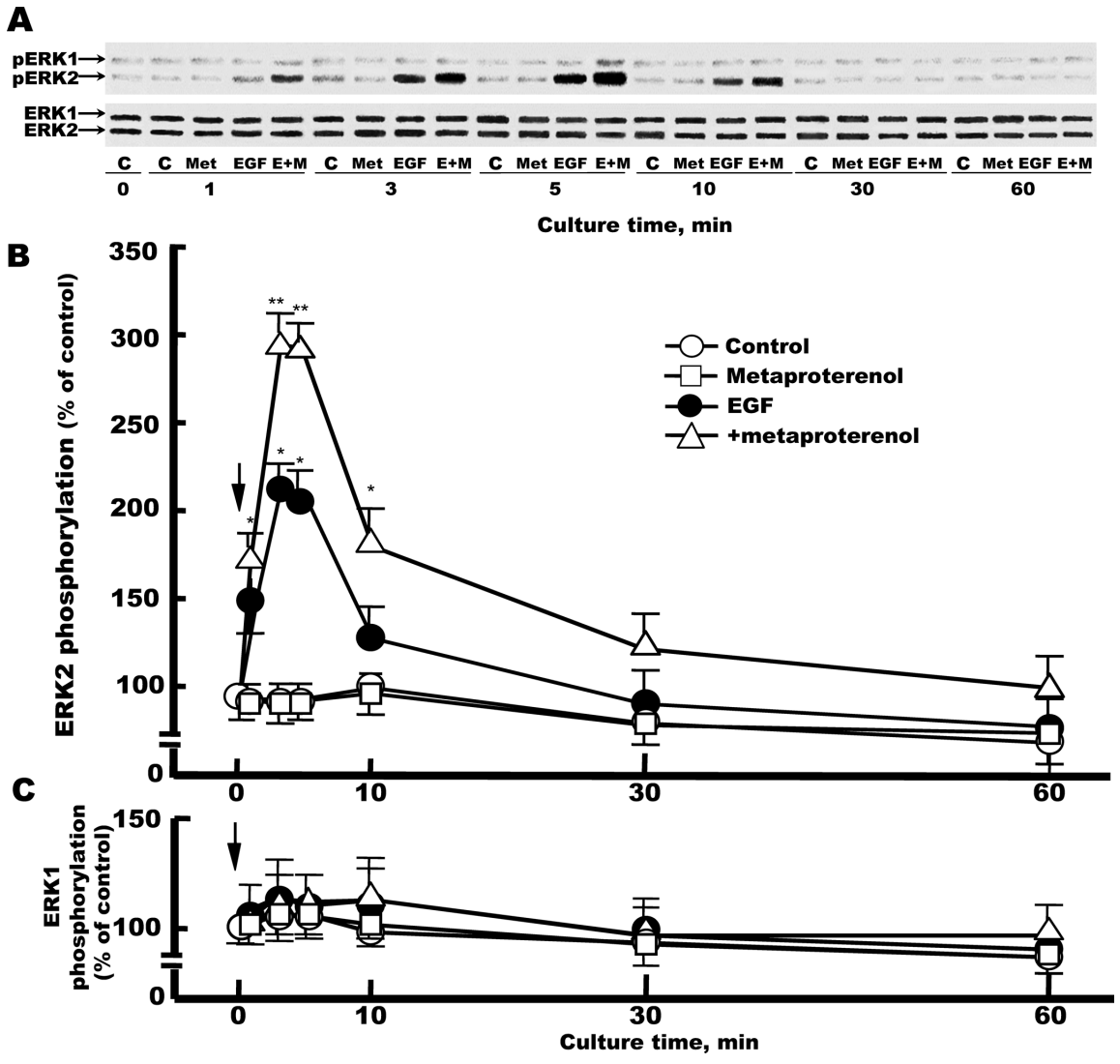

Fig. 1. Time Course and Patterns of ERK Isoform Phosphorylation Induced by EGF in the Absence and/or Presence of Metaproterenol

Isolated hepatocytes were cultured for 3 h, then washed and incubated in either the absence (control; C: medium alone), 10^{-6} M metaproterenol (Met), presence of 20 ng/ml EGF (EGF), or 20 ng/ml EGF with 10^{-6} M metaproterenol (E+M) for the indicated times (min) at 37 °C. Phosphorylated ERK isoforms (pERK1/2) were identified by Western blotting using an anti-pERK antibody, as described in Materials and Methods. The intensity of the M_r 44-kDa and 42-kDa bands, corresponding to pERK1 and pERK2, respectively, was normalized to total ERK (ERK1/2). (A) Representative Western blot; (B) time–course of pERK2 phosphorylation; (C) time–course of pERK1 phosphorylation. Results are expressed as a percentage of the respective control value (mean±S.E.M. of three experiments). $*p<0.05$; $**p<0.01$ compared with respective controls (medium alone).

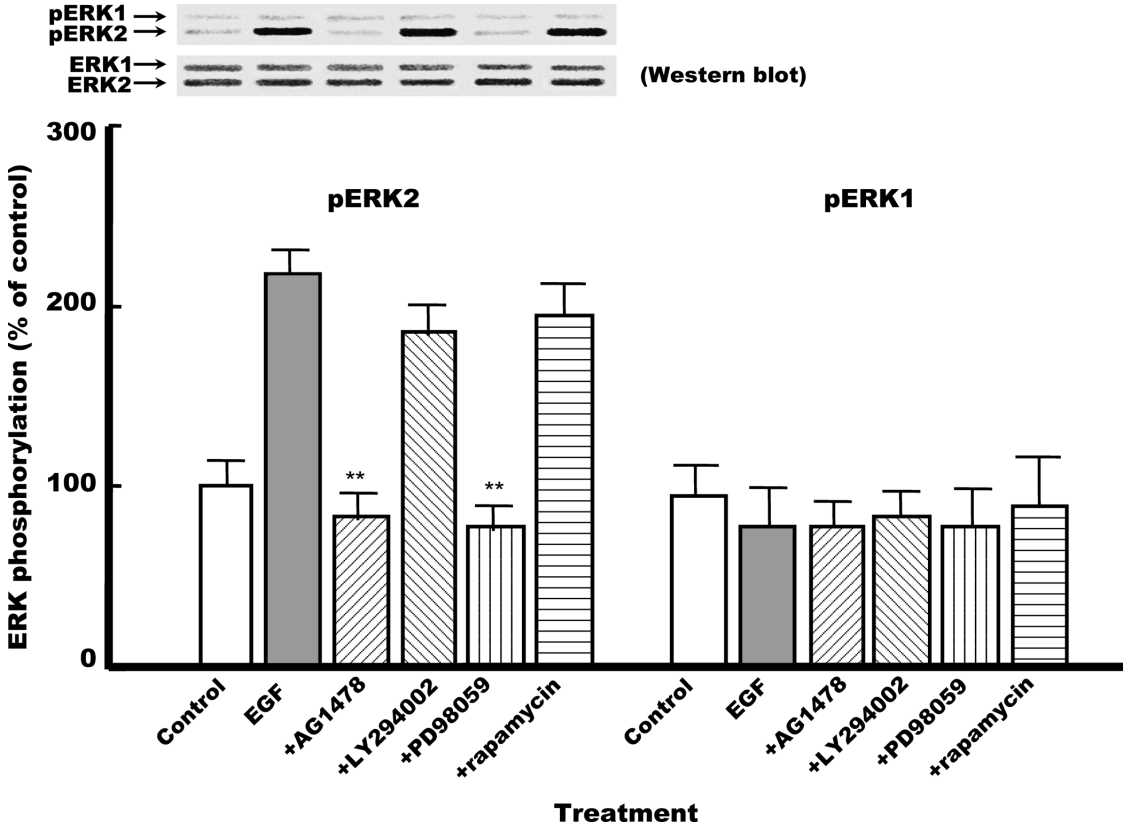

Fig. 2. Effects of Specific Inhibitors of Signal Transducers on EGF Induction of ERK Isoform Phosphorylation

Hepatocytes were stimulated for 5 min with EGF (20 ng/ml) in the presence or absence of specific inhibitors of signal transducers: AG1478 (10^{-7} M), LY294002 (10^{-6} M), PD98059 (10^{-6} M), or rapamycin (10 ng/ml). Phosphorylated ERK isoforms (p44 kDa/pERK1 and p42 kDa/pERK2) were identified by Western blotting using an anti-phospho-ERK antibody as described in Materials and Methods. Typical Western blot images are indicated on the top of figure. Results are expressed as a percentage of the respective control value (mean±S.E.M. of three experiments). ** $p<0.01$ compared with respective EGF-treated controls (EGF alone).

tact.

Isolated hepatocytes were plated onto collagen-coated plastic culture dishes (35 mm diameter) at a density of 3.3×10^4 cells/cm^2 in Williams' medium E containing 5% bovine calf serum and 0.1 nM dexamethasone for 3 h at 37 °C in 5% CO_2 in air. The medium was then changed, and the cells were cultured in serum- and dexamethasone-free Williams' medium E containing EGF (20 ng/ml) with or without α_1-, α_2- and β_2-adrenergic agonists and/or specific effectors or inhibitors of signal transducers for the indicated times at 37 °C.

Measurement of Extracellular Signal-Regulated Kinases 1 and 2 (ERK1/2) Phosphorylation Phosphorylated ERK isoforms (pERK1; P-p44 MAPK and pERK2; P-p42 MAPK) were identified by Western blotting analysis using anti-phospho-ERK1/2 monoclonal antibody as described by Towbin *et al.*[22] Phosphorylated ERK1/2 phosphorylation was normalized to total ERK1/2 levels. Briefly, cultured hepatocytes were washed once with ice-cold phosphate-buffered saline (pH 7.4) and 0.2 ml of lysis buffer (10 mM Tris–HCl buffer (pH 7.4), 150 mM NaCl, 2 mM ethylene glycol bis(2-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), 2 mM dithiothreitol, 1 mM sodium orthovanadate, 1 mM phenylmethylsulphonyl fluoride, 10 μg/ml leupeptin, 10 μg/ml aprotinin) was added, after which the hepatocytes were harvested. After centrifugation at 16300×***g*** for 30 min at 4 °C, cell lysates were denatured in boiling water for 5 min. Samples of the supernatant (30 μg of protein) were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using a 10% acrylamide resolving gel according to the method of Laemmli.[23] After electrophoresis, proteins were transferred to Immobilon-P membranes.

For detection of phosphorylated extracellular-regulated kinase 1 and 2 (pERK1/2), the membranes were immersed in Tris-buffered saline (pH 7.4) containing 1% bovine serum albumin. The membranes were then incubated with an antibody (1 μg/ml) against pERK1/2 and/or ERK1/2, and were washed as described previously.[22] Antibody binding was visualized by incubation with a horseradish peroxidase-conjugated donkey anti-rabbit immunoglobulin G (IgG) secondary antibody (1 : 3000 dilution) followed by enhanced chemiluminescence detection (ECL Kit, Amersham, U.K.). Densitometric analysis was performed using NIH Image version 1.68 for Macintosh. Data were calculated in arbitrary units and are expressed as means±S.E.M.

Cytosolic protein in hepatocytes was quantified by modification of the Lowry procedure using bovine serum albumin as a standard.[24]

Determination of EGF Receptor Tyrosine Kinase Phosphorylation The 175-kDa EGF receptor protein was identified by immunoprecipitation and subsequent immunoblotting with the corresponding anti-phospho-receptor tyrosine kinase antibody according to the protocol of the manufacturer's instructions. In brief, hepatocytes were freshly isolated and seeded at a cell density of 3.3×10^4 cells/cm^2 and cul-

tured in Williams' medium E containing 5% newborn bovine serum. Cultured hepatocytes were washed once with ice-cold phosphate-buffered saline (pH 7.4) and 0.2 ml lysis buffer (20 mM Tris buffer, pH 7.5, 1% Triton X-100, 150 mM NaCl, 1 mM ethylenediamine-*N*,*N*,*N*′,*N*′-tetraacetic acid (EDTA), 1 mM EGTA, 2.5 mM sodium pyrophosphate, 1 mM sodium orthovanadate, 1 mM β-glycerophosphate, phenylmethylsulfonyl fluoride) was added. Cell lysates were obtained by scraping the cells in lysis buffer followed by sonication for 3 min. Cell lysates were then spun down (3000×*g* for 3 min at 4 °C), and the supernatants were denatured in boiling water for 5 min. For immunoblotting analysis using anti-phospho-EGF receptor (Tyr1068) antibody, samples of the supernatant (30 μg/lane) were resolved by SDS-PAGE (7.5% polyacrylamide gel), transferred to polyvinylidene difluoride (PVDF) membrane, and immunoblotted with anti-phosphotyrosine antibodies. Blots were developed using enhanced chemiluminescence following incubation with horseradish peroxidase-conjugated secondary antibodies.[25] The supernatant protein concentration was determined using Lowry's assay method.[24]

Quantification was performed by densitometry after development of the membrane with the enhanced chemiluminescence reagent and exposure to Hyperfilm (Kodak, Japan). Densitometric analysis was performed using the NIH image program version 1.6 for Macintosh. Data were calculated in arbitrary units and are expressed as means±S.E.M. The autodiagram is representative of three experiments using different cell preparations.

Measurement of Hepatocyte DNA Synthesis and Proliferation Hepatocyte DNA synthesis was assessed by measuring by [^{3}H]-thymidine incorporation into acid-precipitable materials.[26] After an initial attachment period of 3 h, the hepatocytes were washed twice with serum-free Williams' medium E and cultured in a medium containing EGF (20 ng/ml) for a further 4 h. The cells were pulsed at 2 h and post-EGF-stimulation for 2 h with [^{3}H]-thymidine (1.0 μCi/well). Incorporation of [^{3}H]-thymidine into DNA was determined as described previously.[9] Hepatocyte protein content was determined by a modified Lowry procedure,[24] using bovine serum albumin as the standard. Data are expressed as dpm/h/mg cellular protein.

The number of nuclei rather than the number of cells was counted using a modified version of the procedure described previously.[9,16]

Statistical Analysis Group comparisons were made by analysis of variance (ANOVA) for unpaired data followed by *post hoc* analysis using Dunnett's multiple comparison tests. Differences of $p<0.05$ were considered to be statistically significant.

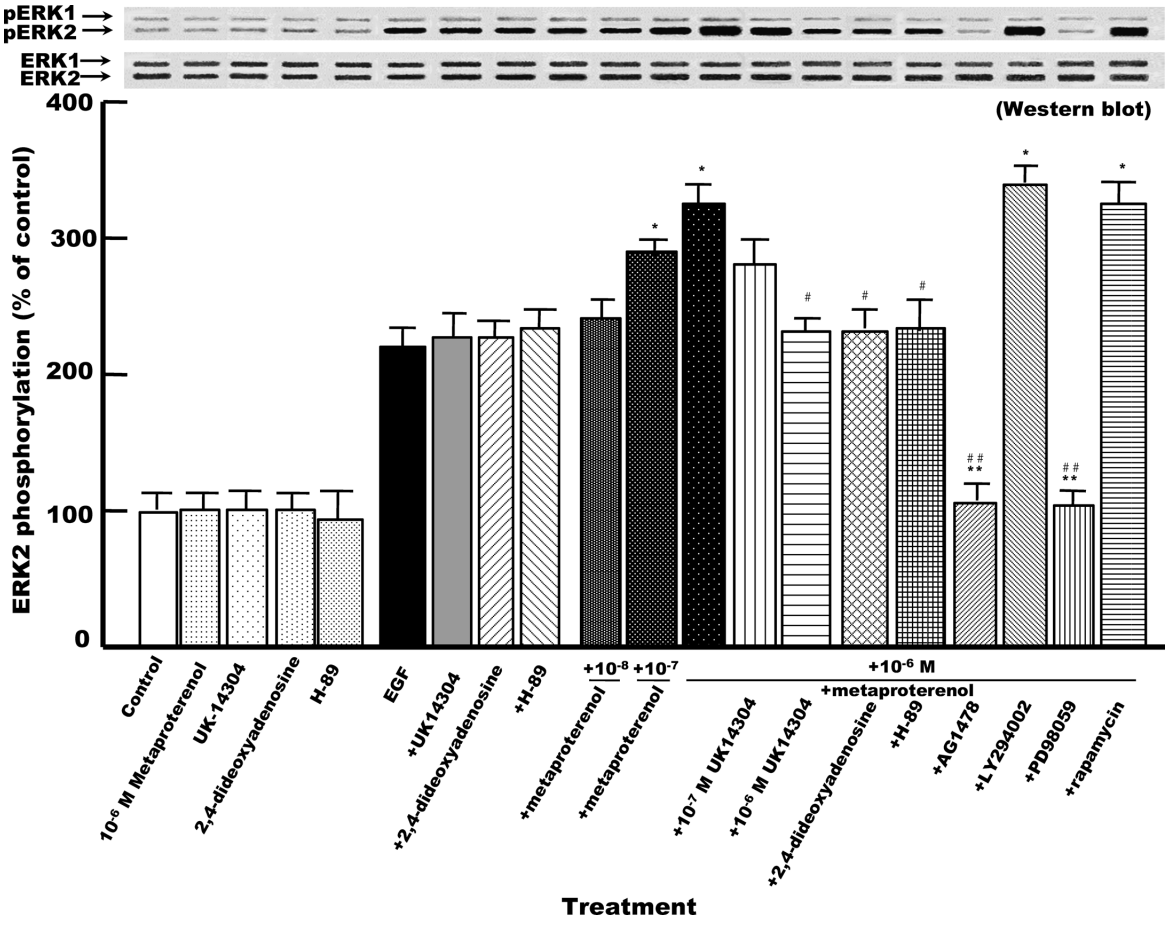

Fig. 3. Effect of Specific Inhibitors of Signal Transducers on Metaproterenol-Induced ERK2 Phosphorylation in the Presence of EGF

Hepatocytes were stimulated for 5 min with or without EGF (20 ng/ml) in the presence or absence of metaproterenol (10^{-8} to 10^{-6} M) and in the presence or absence of the specific signal transducer inhibitors; AG1478 (10^{-7} M), PD98059 (10^{-6} M), LY294002 (10^{-6} M), rapamycin (10 ng/ml), H-89 (10^{-7} M), UK14304, (10^{-7}/10^{-8} M) or 2,4-dideoxyadenosine (10^{-6} M). Phosphorylated ERK isoforms (pERK1 and pERK2) were determined by Western blotting using an anti-phospho-ERK1/2 antibody, as described in Materials and Methods. Typical Western blot images are indicated on the top of figure. Results are expressed as a percentage of the respective control value (mean±S.E.M. of three experiments). $*p<0.05$; $**p<0.01$ compared with respective EGF-treated controls (EGF alone), $^{\#}p<0.05$; $^{\#\#}p<0.01$ compared with EGF plus metaproterenol (10^{-6} M)-treated controls.

RESULTS

Time Course and Patterns of EGF Stimulation of ERK Isoform Phosphorylation, and Their Potentiation by the β_2-Adrenergic Agonist Metaproterenol Figure 1A shows the typical pattern of phospho-ERK1/2 (pERK1/2) in isolated hepatocytes in culture as detected by Western blotting analysis. The phosphorylated ERK2 band (pERK2) was induced after only 1 min, and peaked (about 2-fold increase) between 3 and 5 min, after addition of 20 ng/ml EGF (Figs. 1A, B). ERK1 phosphorylation was not significantly affected by either medium alone (control) or by EGF treatment (Figs. 1A, C).

The time course of the effect of a β_2-adrenergic agonist on ERK1/2 phosphorylation induced by 20 ng/ml EGF was investigated using metaproterenol. Stimulation of hepatocytes with EGF in the presence of metaproterenol (10^{-6} M[27]) induced a more rapid and significant increase in ERK2 phosphorylation than stimulation with EGF alone, reaching a peak at 5 min after addition (about 3-fold increase from baseline) and rapidly declining to basal levels within 30 min (Figs. 1A, B). In contrast, metaproterenol alone did not significantly stimulate ERK2 phosphorylation (Figs. 1A, B). Metaproterenol in the presence of EGF also did not significantly stimulate ERK1 phosphorylation (Figs. 1A, C). These data indicate that metaproterenol (10^{-6} M) specifically potentiated EGF-induced ERK2 phosphorylation.

Effects of Specific Inhibitors of Signal Transducers on EGF-Stimulated ERK1/2 Isoform Phosphorylation In order to characterize the involvement of ERK1/2 in the mitogenic pathway induced by EGF, we investigated the effects of the EGF receptor tyrosine kinase inhibitor AG1478 (10^{-7} M[28]), the PI3 kinase inhibitor LY294002 (10^{-6} M[29]), the MEK inhibitor PD98059 (10^{-6} M[30]) and the mammalian target of rapamycin (mTOR) inhibitor rapamycin (10 ng/ml[31]) on EGF-induction of ERK1/2 phosphorylation. As shown in Fig. 2, the phosphorylation of ERK2 induced by EGF (20 ng/ml) at 5 min was almost completely blocked by AG1478 or PD98059. However, LY294002 and/or rapamycin did not affect EGF-induced ERK2 phosphorylation. Neither EGF (20 ng/ml) nor EGF plus any of the inhibitors induced phosphorylation of ERK1 (Fig. 2).

Effect of Specific Inhibitors of Signal Transducers on Metaproterenol and/or 8-Bromo cAMP-Induced ERK1/2 Isoform Phosphorylation in the Presence of EGF In order to investigate the mechanisms by which the β_2-adrener-

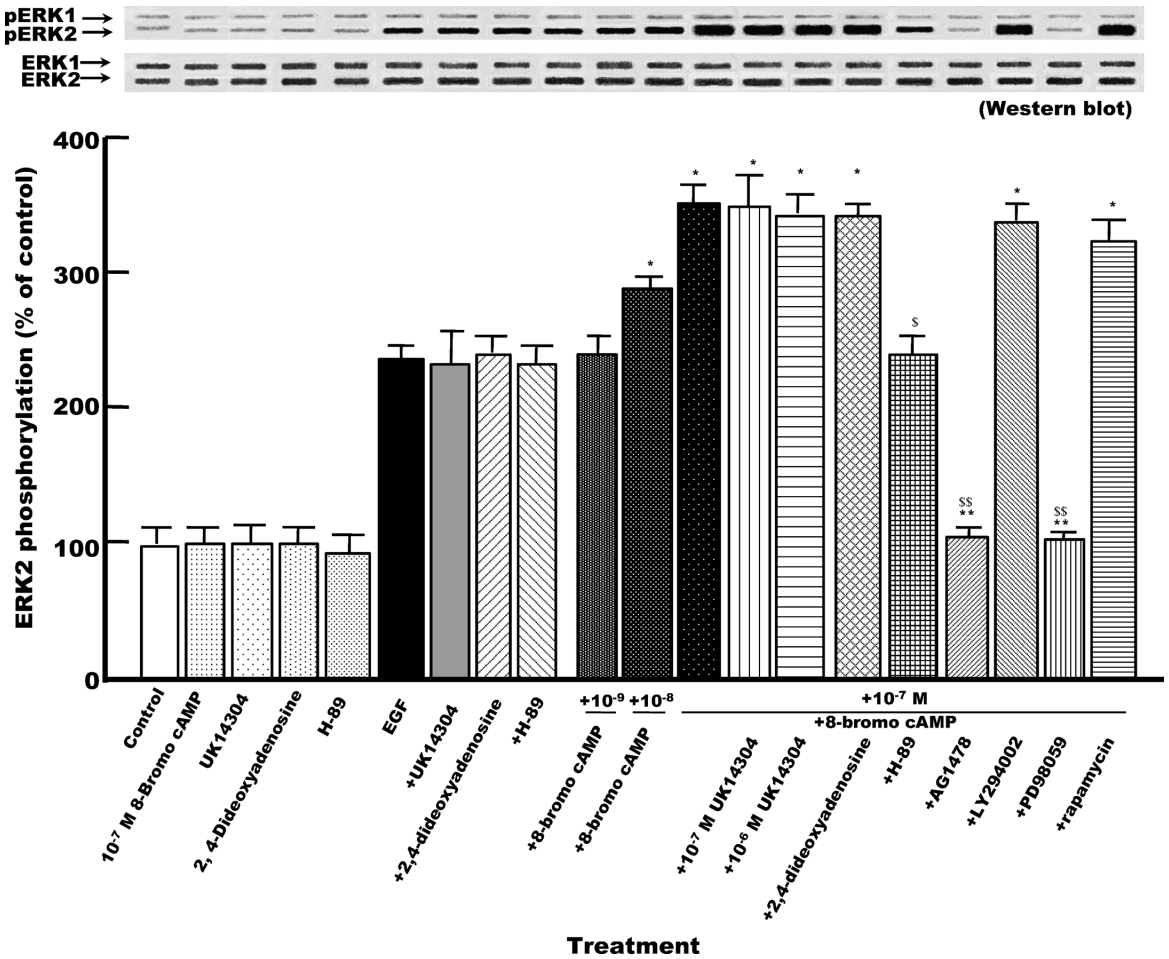

Fig. 4. Effects of Specific Inhibitors of Signal Transducers on 8-Bromo cAMP-Induced ERK2 Phosphorylation in the Presence of EGF

Hepatocytes were stimulated for 5 min with or without EGF (20 ng/ml) in the presence or absence of 8-bromo cAMP (10^{-9}—10^{-7} M) and in the presence or absence of the specific signal transducer inhibitors; AG1478 (10^{-7} M), PD98059 (10^{-6} M), LY294002 (10^{-6} M), rapamycin (10 ng/ml), 2,4-dideoxyadenosine (10^{-6} M) and H-89 (10^{-7} M), UK14304, (10^{-7}/10^{-8} M) or 2,4-dideoxyadenosine (10^{-6} M). Phosphorylated ERK1/2 isoforms (pERK1/2) were determined by Western blotting using an anti-phospho-ERK1/2 antibody, as described in Materials and Methods. Typical Western blot images are indicated on the top of figure. Results are expressed as a percentage of the respective control value (mean±S.E.M. of three experiments). $*p<0.05$; $**p<0.01$ compared with respective EGF-treated controls (EGF alone). $^{\$}p<0.05$; $^{\$\$}p<0.01$ compared with EGF plus 8-bromo cAMP (10^{-7} M)-treated controls.

gic receptor/protein kinase A (PKA) pathway potentiates EGF-induction of ERK2 phosphorylation, we examined the effects of metaproterenol and the cell-permeable cAMP analog 8-bromo cAMP[32] on EGF-induced ERK2 phosphorylation. The dose-dependent potentiation of ERK2 phosphorylation induced by either metaproterenol or 8-bromo cAMP in the presence of EGF was almost completely reduced to control levels by AG1478 (10^{-7} M) and PD98059 (10^{-6} M), but not by LY294002 or rapamycin (Figs. 3, 4). In addition, the α_2-adrenergic agonist UK14304[33] dose-dependently inhibited the metaproterenol-induced potentiation of ERK2 phosphorylation in the presence of EGF (Fig. 3), but not 8-bromo cAMP-induced potentiation (Fig. 4). Furthermore, pretreatment of hepatocytes with the direct AC inhibitor 2,4-dideoxyadenosine (10^{-6} M[34]) blocked the potentiating effect of metaproterenol on the phosphorylation of ERK2 in the presence of EGF, but not the effect of 8-bromo cAMP. In contrast, pretreatment of hepatocytes with the PKA inhibitor H-89 (10^{-7} M[35]) blocked the potentiating effects of metaproterenol and 8-bromo cAMP on the phosphorylation of ERK2 in the presence of EGF. UK14304 (10^{-6} M), metaproterenol (10^{-6} M) or 8-bromo cAMP (10^{-7} M) alone had no significant effect on the phosphorylation of ERK1 or ERK2 (Figs. 3, 4).

Effects of Specific Inhibitors of Signal Transducers on α_1-Adrenergic Agonist Phenylephrine-Induced ERK Isoform Phosphorylation in the Presence of EGF The effects of α_1-adrerenergic agonists on induction of ERK1/2 phosphorylation by 20 ng/ml EGF were investigated using the α_1-adrenergic agonist phenylephrine (10^{-6} M[36]) and 12-*O*-tetradecanoylphorbol-13-acetate (phorbol ester, TPA[37]). When hepatocytes were stimulated with EGF in the presence of phenylephrine (10^{-6} M), and/or TPA (10^{-7} M), significant potentiation of ERK2 phosphorylation was not observed when compared with EGF (20 ng/ml) alone (Fig. 5). EGF-induced ERK2 phosphorylation in the presence of phenylephrine (10^{-6} M) was also completely blocked by AG1478 and PD98059, but was not blocked by LY294002, or rapamycin. In addition, pretreatment of hepatocytes with the phospholipase C (PLC) inhibitor U-73122 (10^{-6} M[38]), an inactive analogue of U-73122, U-73343 (10^{-6} M[38]), or the PKC inhibitor GF109203X (10^{-7} M[39]) did not block the phosphorylation of ERK2 in the presence of EGF. Phenylephrine (10^{-6} M), TPA (10^{-7} M), U-73122 (10^{-6} M), U-73343 (10^{-6} M) or GF109203X alone had no significant effect on the phosphorylation of ERK1 or ERK2 (Fig. 5).

Time Course and Patterns of EGF Stimulation of EGF Receptor Tyrosine Kinase Phosphorylation with or without the β_2-Adrenergic Agonist Metaproterenol Figure 6A shows the typical pattern of phospho-EGF receptor tyrosine kinase (Tyr1068) in isolated hepatocytes in culture as detected by Western blotting analysis. The tyrosine phosphorylation of a 175-kDa protein (EGF receptor tyrosine kinase)

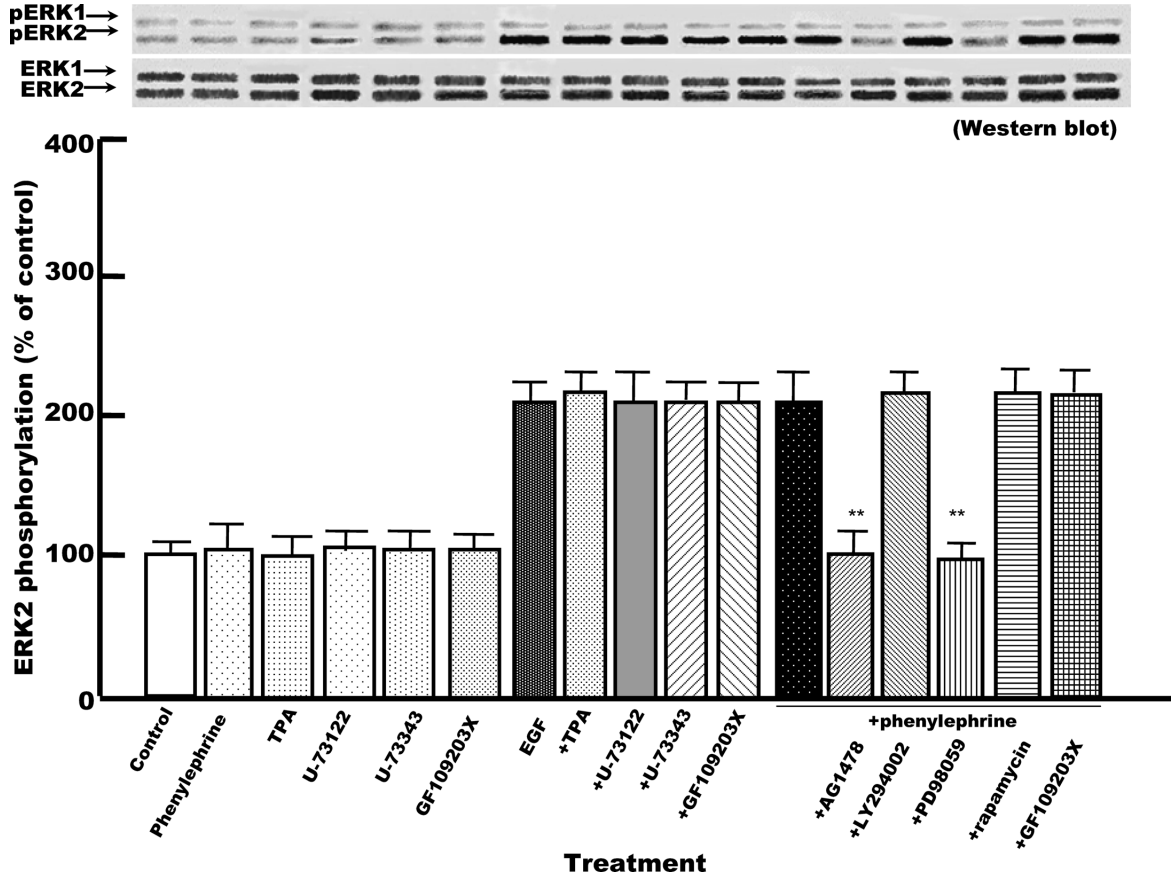

Fig. 5. Effects of Specific Inhibitors of Signal Transducers on Phenylephrine and/or TPA-Induced ERK2 Phosphorylation in the Presence of EGF

Hepatocytes were stimulated for 5 min with or without EGF (20 ng/ml) in the presence or absence of phenylephrine (10^{-6} M), and/or TPA (10^{-7} M) and in the presence or absence of specific inhibitors of the signal transducers; AG1478 (10^{-7} M), PD98059 (10^{-6} M), LY294002 (10^{-6} M), rapamycin (10 ng/ml), U-73122, U-73343 and GF109203X (10^{-7} M). Phosphorylated ERK1/2 isoforms (pERK1/2) were determined by Western blotting using an anti-phospho-ERK1/2 antibody, as described in Materials and Methods. Typical Western blot images are indicated on the top of figure. Results are expressed as a percentage of the respective control value (mean±S.E.M. of three experiments). $**p<0.01$ compared with respective EGF-treated controls (EGF alone).

band (p175-kDa RTK) was induced only 1 min after EGF addition, and peaked about 3 min (about 3-fold increase), and then rapidly declining to basal levels within 10 min after addition of 20 ng/ml EGF (Figs. 6A, B). EGF receptor tyrosine kinase phosphorylation was not significantly affected by medium alone (control) during treatment times (Figs. 6A, B).

The time course of the effect of a β_2-adrenergic agonist on EGF receptor tyrosine kinase phosphorylation induced by 20 ng/ml EGF was investigated using metaproterenol. Metaproterenol (10^{-6} M) had not any effect on hepatocyte EGF receptor tyrosine kinase phosphorylation in the absence or presence of EGF (Figs. 6A, B). These data indicate that metaproterenol (10^{-6} M) did not directly affect EGF receptor tyrosine kinase phosphorylation.

Effect of Specific Inhibitors of Growth-Related Signal Transducers and cAMP-Elevating Agents on EGF Receptor Tyrosine Kinase Phosphorylation To obtain further support for the cross-talk between EGF receptor mediated-tyrosine kinase-induced ERK1/2 kinase activation (ERK1/2 phosphorylation) and α_2- and β_2-adrenergic receptor activities, we examined the effects of specific inhibitors of growth-related signal transducers on EGF receptor tyrosine kinase phosphorylation induced by α_2- and β_2-adrenergic receptor agonists. Figure 7 shows that EGF (20 ng/ml) caused an increase in the tyrosine phosphorylation of a 175-kDa protein (EGF receptor tyrosine kinase) that peaked 3 min after addition, at about 3.0-fold (compared with control). When EGF (20 ng/ml) was added in combination with AG1478 (10^{-7} M), the added AG1478 completely abolished the EGF-induced increase in receptor autophosphorylation (Tyr1068). In contrast, EGF-induced receptor tyrosine kinase activation was not abolished by LY294002 (10^{-6} M), PD98059 (10^{-6} M), rapamycin (10 ng/ml), 2,4-dideoxyadenosine (10^{-6} M), or H-89 (10^{-7} M) treatment. In addition, the α_2-adrenegic receptor agonist UK14304 (10^{-6} M), the β_2-adrenegic receptor agonist metaproterenol (10^{-6} M), the cell-permeable cAMP analog 8-bromo cAMP (10^{-7} M), 2,4-dideoxyadenosine (10^{-6} M) and H-89 (10^{-7} M) had no significant effect on the phosphorylation of EGF receptor tyrosine kinase in the absence of EGF (Fig. 7).

Effects of Specific Inhibitors of Growth-Related Signal-Transducers on Hepatocyte DNA Synthesis and Proliferation Induced by EGF In order to confirm the notion that 20 ng/ml EGF induces hepatocyte DNA synthesis and proliferation through receptor tyrosine kinase, ERK or mTOR phosphorylation, we investigated whether or not AG1478, LY294002, PD98059, and rapamycin are able to inhibit EGF-induced hepatocyte DNA synthesis and proliferation in the presence or absence of metaproterenol or 8-bormo cAMP. As shown in Fig. 8, AG1478 (10^{-7} M), PD98059 (10^{-6} M), and rapamycin (10 ng/ml) almost completely blocked EGF-induced hepatocyte DNA synthesis and proliferation with or without metaproterenol (10^{-6} M) or 8-bormo cAMP (10^{-7} M), but not LY294002 (10^{-6} M). In addition, potentiating effect of metaproterenol (10^{-6} M) on EGF-induced hepatocyte DNA synthesis and proliferation was blocked by 2,4-

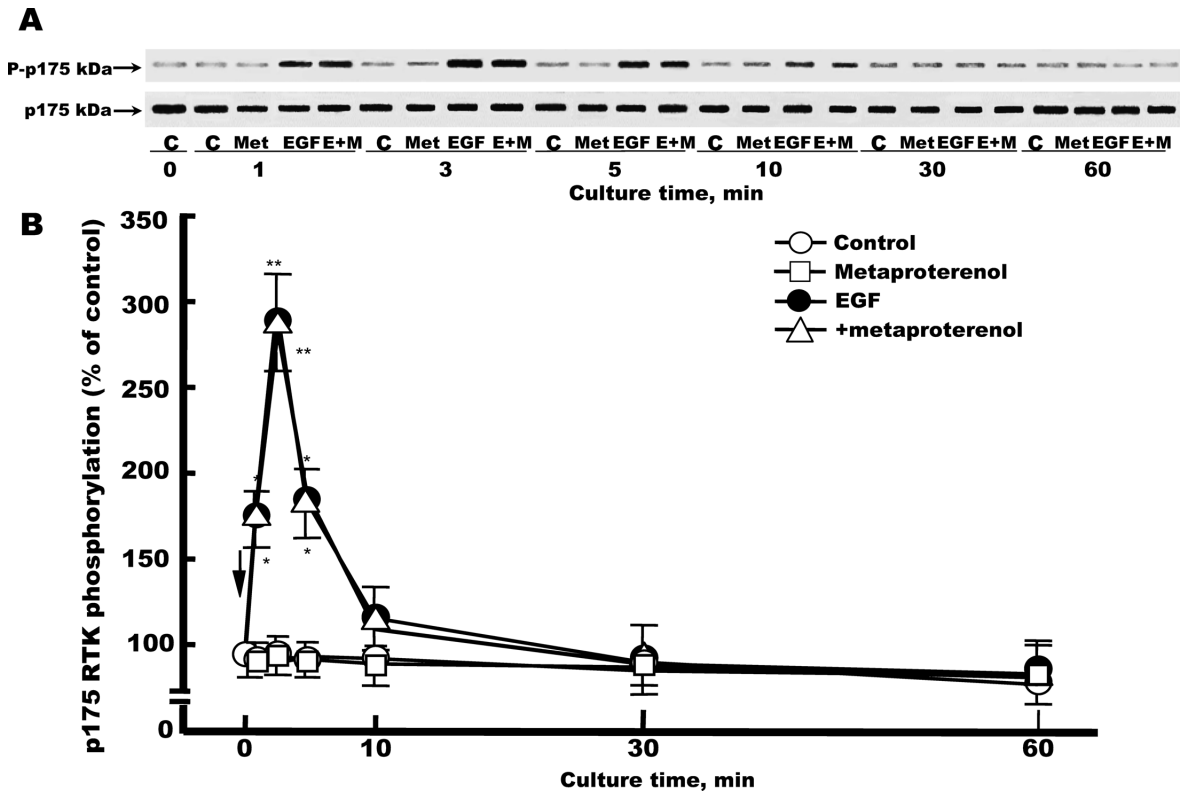

Fig. 6. Time Course and Patterns of EGF Receptor Tyrosine Kinase Phosphorylation Induced by EGF in the Absence or Presence of Metaproterenol

Isolated hepatocytes were cultured for 3 h, then washed and incubated in either the absence (control; C: medium alone), of 10^{-6} M metaproterenol (Met), or presence of 20 ng/ml EGF (EGF), and 10^{-6} M metaproterenol (E+M) for the indicated times (min) at 37 °C. Phosphorylated EGF receptor tyrosine kinase (P-p175 kDa) and total receptor tyrosine kinase protein (p175 kDa) was identified by Western blotting using an anti-phospho-EGF receptor (Tyr1068) antibody, as described in Materials and Methods. The intensity of the M_r 175-kDa band, corresponding to phospho-EGF receptor tyrosine kinase (P-p175 kDa), was normalized to total EGF receptor tyrosine kinase (p175 kDa). (A) typical Western blot; (B) time–course of EGF receptor tyrosine kinase phosphorylation. Results are expressed as a percentage of the respective control value (mean±S.E.M. of three experiments). $*p<0.05$; $**p<0.01$ compared with respective controls.

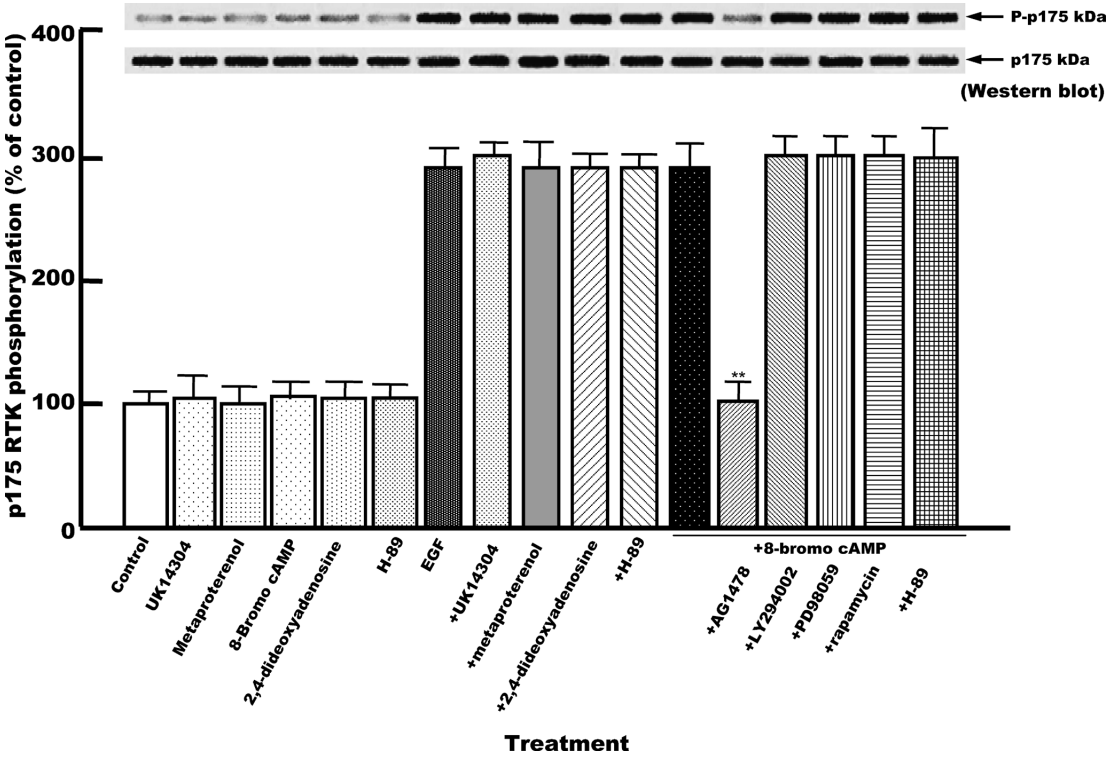

Fig. 7. Effect of Specific Inhibitors of Growth-Related Signal Transducers on EGF Receptor Tyrosine Kinase Phosphorylation

Freshly isolated hepatocytes were plated at a density of 3.3×10^4 cells/cm^2 and cultured as described in the legend to Fig. 1. After a medium change the hepatocytes were cultured with EGF, with or without the indicated specific inhibitors of signal transducers, for 3 min. Phosphorylated EGF receptor tyrosine kinase (P-p175 kDa) and total receptor tyrosine kinase protein (p175 kDa) (upper) were determined by Western blotting analysis as described in Materials and Methods. Typical Western blot images are indicated on the top of figure. The concentrations used were as follows: EGF, 20 ng/ml; UK14304, 10^{-6} M; metaproterenol, 10^{-6} M; 8-bromo cAMP, 10^{-7} M; 2,4-dideoxyadenosine, 10^{-6} M; H-89, 10^{-7} M; AG1478, 10^{-7} M; LY294002, 10^{-6} M; PD98059, 10^{-6} M and rapamycin, 10 ng/ml. The results are expressed as the mean±S.E.M. of three different experiments. $**p<0.01$ compared with respective EGF-treated controls (EGF alone).

dideoxyadenosine (10^{-6} M) and H-89 (10^{-7} M) treatment. In contrast, potentiating effect of 8-bormo cAMP (10^{-7} M) was blocked by H-89 (10^{-7} M), but not 2,4-dideoxyadenosine (10^{-6} M) treatment. Metaproterenol (10^{-6} M) and 8-boromo cAMP alone had not any effect on EGF-induced DNA synthesis and proliferation (Fig. 8).

DISCUSSION

It has been reported that ERK plays an important role in the proliferation of hepatocytes and other cells induced by growth factors and cytokines.[3,14,15,40] The proliferative pathway by which EGF activates the receptor tyrosine kinase (p175 kDa)/MAPK cascade (ERK1/2 pathway) has been described in many normal and transformed cells.[5,9,11,15,40] The ERK pathway is one of the most important and intensively studied signaling pathways.[14,15,41] However, there are few studies regarding EGF-induced activation of ERK1/2 isoforms in primary cultured adult rat hepatocytes. Therefore, we investigated the possible roles of the ERK1/2 isoforms whose phosphorylation was induced by EGF in primary cultures of adult rat hepatocytes.

As shown in Figs. 1 and 2, EGF (20 ng/ml), a concentration that induces hepatocyte DNA synthesis and proliferation,[9] rapidly stimulates ERK2, but not ERK1 phosphorylation. Frémin *et al.* show that continued expression of cyclin-D1 in the G_0/G_1 phase is dependent on ERK2 expression by EGF, and that the ablation of ERK1 influences neither the proliferation capacity of mice hepatocytes in this regulation, *in vivo* and *in vitro*, nor the expression pattern of cyclin-D1.[42] Very recently, Frémin *et al.* show that differentiation is ERK1 and ERK2 dependent, whereas ERK2 knockdown inhibited only proliferation and ERK1 silencing increased survival.[43] In agreement with the recent report, our data indicate that ERK2 play a key role in hepatocyte proliferation.[42—45] Moreover, EGF induction of ERK2 phosphorylation in hepatocytes was almost completely blocked by specific inhibitors of signal transducers, such as the EGF receptor tyrosine kinase inhibitor AG1478 and the MEK inhibitor PD98059, but not by LY294002 (PI3 kinase inhibitor) or rapamycin (mTOR inhibitor) (Fig. 2). We have previously reported that EGF induced hepatocyte DNA synthesis and proliferation within 4 h of serum-free culture.[9] The hepatocytes DNA synthesis and proliferation appeared to be dependent on the concentrations of dexamethasone in the culture medium.[46] The EGF effects were almost completely blocked by AG1478, PD98059, and rapamycin, but not by LY294002 (Fig. 8). These results suggest are consistent with a previous report that signal transducers such as the EGF-receptor tyrosine kinase and ERK2 play an essential role in the mitogenic activity induced by EGF under these experimental conditions.[9] The present results are also consistent with reports that ERK2 acts upstream of mTOR/p70S6K and downstream of the EGF receptor tyrosine kinase in primary cultures of adult rat hepatocytes.[9,15,47,48]

There have been very few studies regarding adrenergic agonist-induced regulation of ERK1/2 phosphorylation in hepatocytes. Therefore, we examined whether α_1-, α_2- and

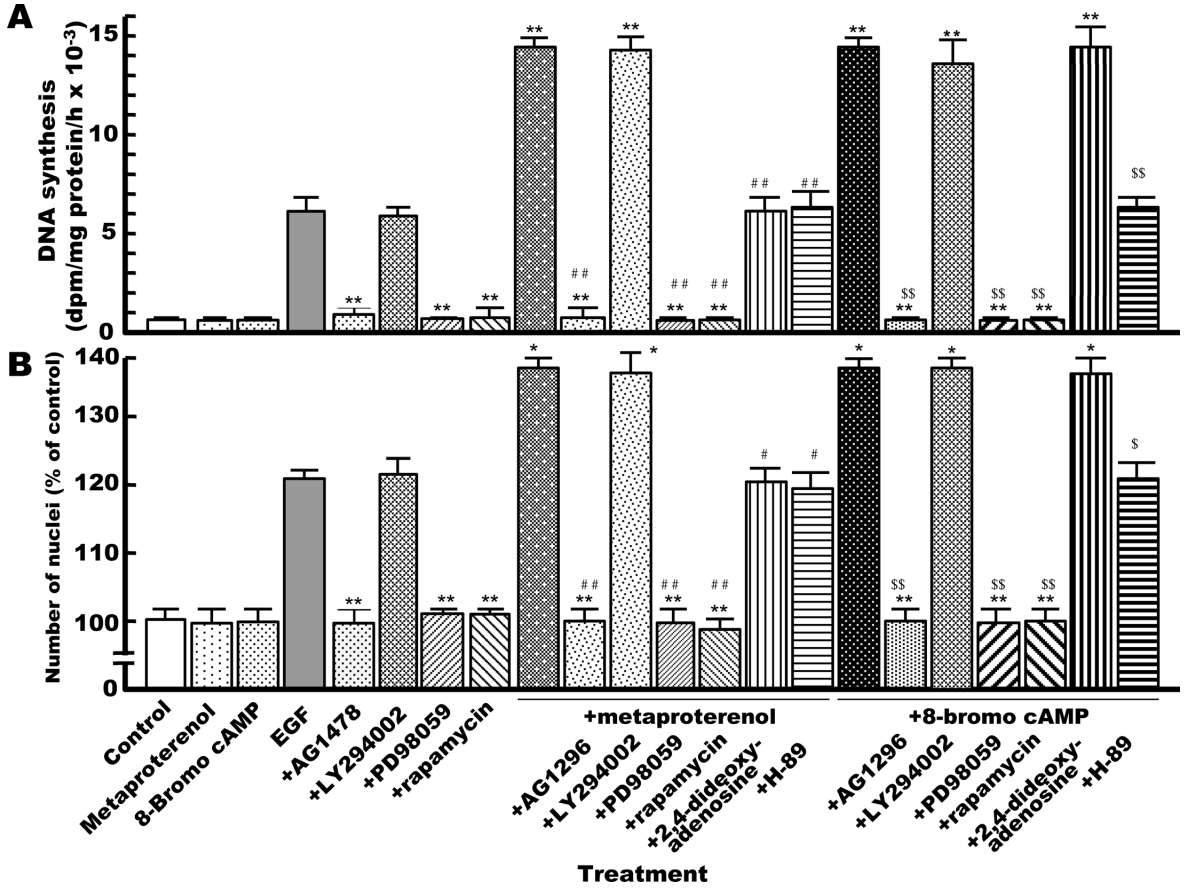

Fig. 8. Effects of Specific Inhibitors of Growth-Related Signal Transducers on Hepatocyte DNA Synthesis and Proliferation Induced by EGF in the Presence or Absence of Metaproterenol or 8-Bormo cAMP

Hepatocytes were plated at a density of 3.3×10^4 cells/cm^2 and cultured as described in the legend to Fig. 1. Specific signal-transducer inhibitors were added without or with 20 ng/ml EGF immediately after the medium change, and the cells were cultured for a further 4 h. The concentrations were as follows: metaproterenol, 10^{-6} M; 8-boromo cAMP, 10^{-7} M; AG1478, 10^{-7} M; LY294002, 10^{-6} M; PD98059, 10^{-6} M; and rapamycin, 10 ng/ml; 2,4-dideoxyadenosine, 10^{-6} M; H-89, 10^{-7} M. The rate of hepatocyte DNA synthesis is expressed as dpm/mg protein/h (A). Hepatocyte proliferation is expressed as the percent increase in total number of nuclei compared with the control culture (B). The results are expressed as means±S.E.M. of three independent experiments. $*p<0.05$, $**p<0.01$ compared with the respective control. $\#p<0.05$; $\#\#p<0.01$ compared with EGF plus metaproterenol (10^{-6} M)-treated controls (EGF+metaproterenol). $\$p<0.05$; $\$\$p<0.01$ compared with EGF plus 8-bromo cAMP (10^{-7} M)-treated controls (EGF+8-bromo cAMP).

β_2-adrenergic agonists modulate the EGF-induced changes in ERK2 phosphorylation. We found that EGF-induced hepatocyte ERK2 phosphorylation was enhanced by metaproterenol (10^{-6} M), a β_2-adrenergic receptor agonist and an indirect adenylate cyclase (AC) activator (Figs. 1, 3). In contrast, metaproterenol had no significant effects on hepatocyte ERK1/2 phosphorylation in the absence of EGF (Fig. 3). The enhancing effects of metaproterenol in the presence of EGF were inhibited by α_2-adrenegic receptor agonist UK14304 (10^{-6} M; an indirect AC inhibitor and decreases cAMP), 2,4-dideoxyadenosine (10^{-7} M; a direct AC inhibitor) and the PKA inhibitor H-89, thus suggesting the involvement of PKA in the potentiating effects of metaproterenol (Fig. 3). The involvement of PKA in the effects of metaproterenol is supported by our previous results, which indicated that metaproterenol-induced potentiation of hepatocyte DNA synthesis and proliferation in the presence of EGF (20 ng/ml) was completely inhibited by a specific PKA inhibitor, H-89 (Fig. 8).

However, the role of the second messenger, cAMP, in the control of hepatocyte DNA synthesis and proliferation remains uncertain.[49—51] Cyclic AMP can either stimulate or inhibit DNA synthesis depending on culture conditions.[51—53] For example, Crespo *et al.* reported that isoproterenol treatment of COS-7 cells had an acute stimulatory and chronic inhibitory effect on ERK2 activity, the former being mediated by G protein bg subunits and the latter by G protein a subunit *via* PKA.[53] We demonstrated that the cell-permeable cAMP analog, 8-bromo cAMP also enhanced the ERK2 phosphorylation and hepatocyte proliferation induced by EGF (Figs. 4, 8). These results indicate that there is cross-talk between the β_2-adrenergic receptor/AC/cAMP pathway and the EGF receptor/ERK pathway, which potentiates hepatocyte growth. Because both metaproterenol- and 8-bromo cAMP-induced potentiation of ERK2 phosphorylation in the presence of EGF were completely inhibited by AG1478 and PD98059, but not by LY294002, or rapamycin, the cross-talk mediated by the β_2-adrenergic pathway may occur upstream of ERK2.

In contrast, EGF-induction of hepatocyte ERK2 phosphorylation was not enhanced by a maximal dose of phenylephrine (10^{-6} M), α_1-adrenergic receptor agonist (an indirect PLC/PKC activator), TPA (10^{-7} M), a synthetic analog of diacylglycerol (a direct activator of PKC), U-73122 (10^{-6} M), a phospholipase C (PLC) inhibitor, and/or the protein kinase C (PKC) inhibitor GF109203X (10^{-7} M) (Fig. 7). These results indicate that the α_1-adrenergic receptor/PLC/PKC pathway is not involved in cross-talk with the EGF receptor/ERK pathway in order to potentiate hepatocyte growth. The results are

different from those of HGF. Accordingly, results were obtained as to the hepatocyte DNA synthesis and proliferation induced by EGF with or without phenylephrine (10^{-6} M).[9)]

To obtain further support for the cross-talk between EGF receptor mediated-tyrosine kinase-induced ERK2 kinase activation (ERK2 phosphorylation) and α_2- and β_2-adrenergic receptor activities, we examined the effects of specific inhibitors of growth-related signal transducers on EGF receptor tyrosine kinase phosphorylation induced by α_2- and β_2-adrenergic receptor agonists. Figure 6 and 7 shows that EGF (20 ng/ml) caused an increase in the tyrosine phosphorylation of a 175-kDa protein (EGF receptor tyrosine kinase) that peaked 3 min after addition, at about 3.0-fold (compared with control). When EGF (20 ng/ml) was added in combination with AG1478 (10^{-7} M), the added AG1478 completely abolished the EGF-induced increase in receptor autophosphorylation (Tyr1068). In contrast, EGF-induced receptor tyrosine kinase activation was not abolished by LY294002 (10^{-6} M), PD98059 (10^{-6} M), rapamycin (10 ng/ml), a specific AC inhibitor, 2,4-dideoxyadenosine (10^{-6} M), or a specific PKA inhibitor, H-89 (10^{-7} M) treatment. In addition, neither the α_2-adrenergic receptor agonist UK14304 (10^{-6} M), the β_2-adrenergic receptor agonist metaproterenol (10^{-6} M) nor the cell-permeable cAMP analog 8-bromo cAMP (10^{-7} M), had any effect on EGF-induced receptor tyrosine kinase activation (Fig. 7).

Therefore, the cross-talk between the α_2- and β_2-adrenergic pathway and EGF signaling may influence signals such as Ras, Raf and MEK that lie downstream of the EGF receptor kinase but upstream of ERK activation.[40,54—56)] In mammals, there are three Raf proteins; Raf-1 (or c-Raf), A-Raf and B-Raf.[57)] Recent reports have produced indirect, but strong evidence for a branching of signals at the level of Raf, in particular Raf-1, which is the focus of the majority of Raf research.[40,55,56)] Therefore, we have done some experiments concerning possible involvement of Raf. The results indicate that EGF-induction of hepatocyte Raf-1 phosphorylation was not enhanced by a maximal dose of metaproterenol (10^{-6} M), and 8-bromo cAMP (10^{-7} M) (data not shown). Hence, participation of other Raf (A-Raf, B-Raf) and/or MEK is currently investigating in our laboratory using primary cultures of adult rat hepatocytes.

The combined data suggest the potential scheme of cross-talk between the EGF receptor/ERK2 pathway and the α_2- and β_2-adrenergic receptor/PKA pathway that is shown in Fig. 9.

In conclusion, the present study demonstrates that cross-talk signals induced by extracellular β_2-adrenoceptor agonists such as metaproterenol potentiate EGF-induced ERK2 phosphorylation, and α_2-adrenoceptor agonists, such as UK14304, attenuate these β_2-adrenoceptor agonist-induced potentiating effects on EGF-induced ERK2 phosphorylation in primary cultured adult rat hepatocytes. Furthermore, the cross-talk signaling may occur downstream of EGF receptor tyrosine kinase, and upstream of MEK/ERK and mTOR/p70 S6K (Fig. 9). The present findings support the notion that endogenous catecholamine-induced potentiation of hepatocyte DNA synthesis and proliferation in the presence of EGF plays an important role in activation of the ERK2 cascade during liver regeneration after partial hepatectomy or in liver necrosis caused by toxic chemicals *in vivo*.[1,3,4)]

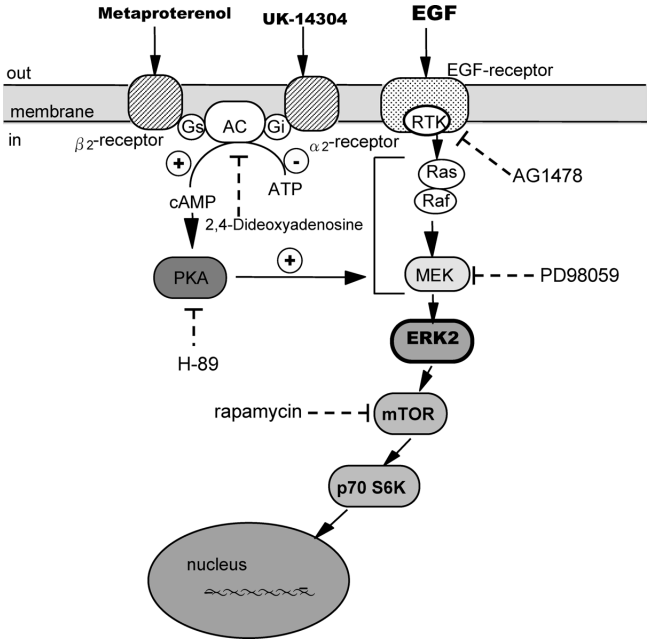

Fig. 9. Potential Model of Cross-Talk between the EGF Receptor/ERK2 Pathway and α_2- or β_2-Adrenergic Receptor-Mediated Pathways

Abbreviations: EGF, epidermal growth factor; TK, receptor tyrosine kinase; MEK, MAP kinase kinase (upstream of ERK[30)]); ERK2, extracellular-signal regulated kinase 2 (p42 MAPK); mTOR, mammalian target of rapamycin; p70S6K, p70 ribosomal protein S6 kinase (downstream of mTOR[31)]); AC, adenylate cyclase; PKA, protein kinase A. → or ⊕, stimulation; – – ⊣ or ⊖, inhibition.

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REFERENCES

1) Noguchi S., Ohba Y., Oka T., *J. Endocrinol.*, **128**, 425—431 (1991).
2) Senaldi G., Shaklee C. L., Simon B., Rowan C. G., Lacey D. L., Hartung T., *Hepatology*, **27**, 1584—1591 (1998).
3) Talarmin H., Rescan C., Cariou S., Glaise D., Zanninelli G., Bilodeau M., Loyer P., Guguen-Guillouzo C., Baffet G., *Mol. Cell. Biol.*, **19**, 6003—6011 (1999).
4) Kimura M., Moro T., Motegi H., Maruyama H., Sekine M., Okamoto H., Inoue H., Sato T., Ogihara M., *Eur. J. Pharmacol.*, **579**, 357—364 (2008).
5) Takai S., Nakamura T., Komi N., Ichihara A., *J. Biochem.*, **103**, 848—852 (1988).
6) Hoffmann R., Baillie G. S., MacKenzie S. J., Yarwood S. J., Houslay M. D., *EMBO J.*, **15**, 893—903 (1999).
7) Abud H. E., Watson N., Heath J. K., *Exp. Cell Res.*, **303**, 252—262 (2005).
8) Li M., Liang C. G., Xiong B., Xu B. Z., Lin S. L., Hou Y., Chen D. Y., Schatten H., Sun Q. Y., *Domest. Anim. Endocrinol.*, **34**, 360—371 (2008).
9) Kimura M., Ogihara M., *Eur. J. Pharmacol.*, **324**, 267—276 (1997).
10) Danielsen A. J., Maihle N. J., *Growth Factors*, **20**, 1—15 (2002).
11) Komurasaki T., Toyoda H., Uchida D., Nemoto N., *Growth Factors*, **20**, 61—69 (2002).
12) Lee M. Y., Park S. H., Lee Y. J., Heo J. S., Lee J. H., Han H. J., *Am. J. Physiol. Gastrointest. Liver Physiol.*, **291**, G744—G750 (2006).
13) Kimura M., Ogihara M., *J. Pharmacol. Exp. Ther.*, **282**, 1146—1154 (1997).
14) Katz M., Amit I., Yarden Y., *Biochim. Biophys. Acta*, **1773**, 1161—1176 (2007).
15) Mebratu Y., Tesfaigzi Y., *Cell Cycle*, **8**, 1168—1175 (2009).
16) Nakamura T., Tomomura A., Kato S., Noda C., Ichihara A., *J. Biochem.*, **96**, 127—136 (1984).

17) Dajani O. F., Sandnes D., Melien O., Rezvani F., Nilssen L. S., Thoresen G. H., Christoffersen T., *J. Cell. Physiol.*, **180**, 203—214 (1999).
18) Kimura M., Okamoto H., Ogihara M., *J. Pharmacol. Sci.*, **103**, 398—407 (2007).
19) Refsnes M., Sandnes D., Melien O., Sand T. E., Jacobsen S., Christoffersen T., *FEBS Lett.*, **164**, 291—298 (1983).
20) Okajima F., Ui M., *Arch. Biochem. Biophys.*, **230**, 640—651 (1984).
21) Seglen P. O., *Methods Cell Biol.*, **13**, 29—83 (1976).
22) Towbin H., Staehelin T., Gordon J., *Proc. Natl. Acad. Sci. U.S.A.*, **76**, 4350—4354 (1979).
23) Laemmli U. K., *Nature* (London), **227**, 680—685 (1970).
24) Lee M. B., Paxman S., *Anal. Biochem.*, **47**, 184—192 (1972).
25) Rojas M., Yao S., Lin Y. Z., *J. Biol. Chem.*, **271**, 27456—27461 (1996).
26) Morley C. G. D., Kingdon H. S., *Anal. Biochem.*, **45**, 298—305 (1972).
27) Ogihara M., *Biol. Pharm. Bull.*, **19**, 752—757 (1996).
28) Levitzki A., Gazit A., *Science*, **267**, 1782—1788 (1995).
29) Vlahos C. J., Matter W. F., Hui K. Y., Brown R. F., *J. Biol. Chem.*, **269**, 5241—5248 (1994).
30) Alessi D. R., Cuenda A., Cohen P., Dudley D. T., Saltiel A. R., *J. Biol. Chem.*, **270**, 27489—27494 (1995).
31) Chung J., Kuo C. J., Crabtree G. R., Blenis J., *Cell*, **69**, 1227—1236 (1992).
32) Derubertis F. R., Zenser T., *Biochim. Biophys. Acta*, **428**, 91—103 (1976).
33) Remaury A., Larrouy D., Daviaud D., Rouot B., Paris H., *Biochem. J.*, **292**, 283—288 (1993).
34) Holgate S. T., Lewis R. A., Austen K. F., *Proc. Natl. Acad. Sci. U.S.A.*, **77**, 6800—6804 (1980).
35) Zuscik M. J., Puzas J. E., Rosier R. N., Gunter K. K., Gunter T. E., *Arch. Biochem. Biophys.*, **315**, 352—361 (1994).
36) Kajiyama Y., Ui M., *Cell. Signal.*, **10**, 241—251 (1998).
37) Smith K. B., Losonczy I., Sahai A., Pannerselvam M., Fehnel P., Salomon D. S., *J. Cell. Physiol.*, **117**, 91—100 (1983).
38) Thompson A. K., Mostafapour S. P., Denlinger L. C., Bleasdale J. E., Fisher S. K., *J. Biol. Chem.*, **266**, 23856—23862 (1991).
39) Toullec D., Pianetti P., Coste H., Bellevergue P., Grand-Perret T., Ajakane M., Baudet V., Boissin P., Boursier E., Loriolle F., Duhamel L., Charon D., Kirilovsky J., *J. Biol. Chem.*, **266**, 15771—15781 (1991).
40) Meloche S., Pouysségur J., *Oncogene*, **26**, 3227—3239 (2007).
41) Seger R., Krebs E. G., *FASEB J.*, **9**, 726—735 (1995).
42) Frémin C., Ezan F., Boisselier P., Bessard A., Pagès G., Pouysségur J., Baffet G., *Hepatology*, **45**, 1035—1045 (2007).
43) Frémin C., Ezan F., Guegan J. P., Gaillouste L., Trotard M., Seyec J. L., Theret N., Langouët S., Baffet G., *J. Cell. Physiol.* (2011), in press.
44) Cárcamo-Orive I., Tejados N., Delgado J., Gaztelumendi A., Otaegui D., Lang V., Trigueros C., *Exp. Cell Res.*, **314**, 1777—1788 (2008).
45) Shukla A., Hillegass J. M., Macpherson M. B., Beuschel S. L., Vacek P. M., Butnor K. J., Pass H. I., Carbone M., Testa J. R., Heintz N. H., Mossman B. T., *Int. J. Cancer*, **129**, 1075—1086 (2011).
46) Kimura M., Moteki H., Ogihara M., *Biol. Pharm. Bull.*, **34**, 682—687 (2011).
47) Ostrowski J., Woszczynski M., Kowalczyk P., Trzeciak L., Hennig E., Bomsztyk K., *J. Hepatol.*, **32**, 965—974 (2000).
48) Downward J., *Nature* (London), **371**, 378—379 (1994).
49) Brønstad G. O., Sand T. E., Christoffersen T., *Biochim. Biophys. Acta*, **763**, 58—63 (1983).
50) Mahler S. M., Wilce P. A., *J. Cell. Physiol.*, **136**, 88—94 (1988).
51) Spector M. S., Auer K. L., Jarvis W. D., Ishac E. J., Gao B., Kunos G., Dent P., *Mol. Cell. Biol.*, **17**, 3556—3565 (1997).
52) Morgan N. G., Blackmore P. F., Exton J. H., *J. Biol. Chem.*, **258**, 5103—5109 (1983).
53) Crespo P., Cachero T. G., Xu N., Gutkind J. S., *J. Biol. Chem.*, **270**, 25259—25265 (1995).
54) Ginès P., Li X., Brown S. E., Nakamura T., Guzelian P. S., Heasley L. E., Schrier R. W., Nemenoff R. A., *Hepatology*, **23**, 1167—1173 (1996).
55) Cox A. D., Der C. J., *Cancer Biol. Ther.*, **1**, 599—606 (2002).
56) Panet R., Eliash M., Atlan H., *J. Cell. Physiol.*, **206**, 578—585 (2006).
57) Hagemann C., Rapp U. R., *Exp. Cell Res.*, **253**, 34—46 (1999).