Activation of Extracellular-Signal Regulated Kinase by Platelet-Derived Growth Factor Is Potentiated by Phenylephrine in Primary Cultures of Adult Rat Hepatocytes

Hajime MOTEKI, Mitsutoshi KIMURA,* and Masahiko OGIHARA

Department of Clinical Pharmacology, Faculty of Pharmaceutical Sciences, Josai University; 1–1 Kayakaidai, Sakado, Saitama 350–0295, Japan. Received November 20, 2010; accepted March 30, 2011; published online April 14, 2011

We investigated the effects of the α-adrenergic agonist phenylephrine on platelet-derived growth factor (PDGF)-stimulated extracellular signal-regulated kinase (ERK) in primary cultures of adult rat hepatocytes. Hepatocytes were isolated and cultured with PDGF (10 ng/ml) and/or α-adrenergic agonist. Phosphorylated ERK isoforms (ERK1 and ERK2) were detected by Western blotting analysis using anti-phospho mitogen-activated protein kinase (MAPK) antibody. PDGF stimulated phosphorylation of ERK2 (42 kDa MAPK) by 2.0-fold within 3—5 min. The PDGF-induced ERK activation was abolished by AG1296 (10⁻⁶ M) or LY294002 (10⁻⁷ M) treatment. MAPK kinase inhibitor, PD98059 (10⁻⁶ M), completely inhibited the PDGF-induced increase in ERK activity. In addition, PDGF-induced mammalian target of rapamycin activity was completely inhibited by AG1296, LY294002, PD98059, or rapamycin treatment. Phenylephrine alone showed no effects on ERKs, but significantly increased phosphorylation of ERK2 induced by PDGF. Moreover, a synthetic analog of diacylglycerol (DG), phorbol 12-myristate 13 acetate (TPA; 10⁻⁷ M), potentiated PDGF-induced ERK2 phosphorylation, while ionomycin had no effect (10⁻⁶ M). The effects of phenylephrine and TPA were antagonized by the phospholipase C (PLC) inhibitor U73122 (10⁻⁷ M), and the protein kinase C (PKC) inhibitor GF109203X (10⁻⁷ M), respectively. Accordingly, PDGF-induced DNA synthesis and proliferation in the presence or absence of phenylephrine or TPA were completely inhibited by AG1296, LY294002, PD98059, or rapamycin treatment. These results suggest that activation of PLC/PKC by phenylephrine represent an indirect positive regulatory mechanism for stimulating ERK induced by 10 ng/ml PDGF.

Key words extracellular-signal regulated kinase; platelet-derived growth factor; cross-talk; α₁-adrenergic agonist; hepatocyte

Platelet-derived growth factor (PDGF) is a potent stimulator of the proliferation by connective tissue cells, such as fibroblasts, smooth muscle cells and hepatic stellate cells, and may play a role in liver regeneration. PDGF-BB is reported to induce smooth muscle cells proliferation and cell migration more effectively than PDGF-AA or -AB. PDGF appears to trigger liver regeneration after partial hepatectomy and chemical-induced acute liver cell necrosis in vivo. The response of adult rat hepatocytes to PDGF has also been investigated extensively with respect to DNA synthesis and proliferation in vitro. The receptor for PDGF, similarly to the receptors for epidermal growth factor (EGF), insulin and hepatocyte growth factor (HGF), contains intrinsic tyrosine kinase activity. The mitogenic effects of PDGF are reportedly mediated through the activation of tyrosine kinase-linked receptors. However, little is known about which of the signals originating in the plasma membrane are involved in the induction of hepatocyte DNA synthesis and proliferation by PDGF. We recently reported that PDGF is able to rapidly stimulate hepatocyte DNA synthesis and proliferation during short-term culture. In addition, the hepatocyte DNA synthesis and proliferation produced by PDGF was not inhibited depending on initial plating density. Furthermore, we found that hepatocyte DNA synthesis and proliferation by PDGF were potentiated by both α₁-adrenergic agonists, but not β₁-adrenergic agonists.

More recently, the signal transduction pathways activated in response to PDGF in hepatocytes and other cell types have become more clearly understood. Using specific inhibitors of signal transducers, we pharmacologically demonstrated that receptor tyrosine kinase, phosphoinositide 3-kinase (PI3K), phospholipase C (PLC) and ribosomal p70 S6 kinase (p70S6K) activities are essential for PDGF-induced DNA synthesis and proliferation in primary cultures of adult rat hepatocytes. In addition, extracellular-signal regulated kinase (ERK)1/2, which is the same as mitogen-activated protein kinase (p42 and p44 MAPK), is now known to be activated in response to a large number of mitogenic stimuli, and the enzyme is a key participant in the response to various growth factors and cytokines. To better understand the PDGF-mediated signaling pathway, we investigated whether activation of ERK isoforms, ERK1 and ERK2, is involved in PDGF-induced DNA synthesis and proliferation in primary cultures of adult rat hepatocytes.

On the other hand, 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 receptor; β₁- and β₂-adrenergic receptors stimulate adenylate cyclase, while α₁-adrenergic receptors inhibit its activity. α₁-Adrenergic receptors are related to phospholipase C (PLC) activation and subsequent increases in inositolphosphate turnover and diacylglycerol production, and protein kinase C (PKC) activation. There have been few studies regarding the adrenergic regulation of ERK activity induced by growth factors in liver cells. Therefore, we examined whether α₁- and β₁-adrenergic agonists can modulate the PDGF-induced activity of ERK isoforms, as there are indications that hepatocyte DNA synthesis and proliferation are modified by α₁-adrenergic receptor-mediated responses in the presence of PDGF.

The results obtained here indicate that PDGF rapidly phosphorylates one of the ERK isoforms, ERK2 (p42 MAPK),...
but not ERK1 (p44 MAPK). Moreover, phenylephrine poten-
tiated the ERK2 phosphorylation induced by PDGF. The
physiological significance of cross-talk between the PDGF
signaling pathway and α1-adrenergic receptor-mediated path-
way 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.): AG1296 (N-
[3-chlorophenyl]-6,7-dimethoxy-4-quinazolinaminil); apro-
tinin; 8-bromo-cAMP; dexamethasone; GF109203X hy-
drochloride (2-[(1-3-dimethylaminopropyl)-1H-indol-3-yl]-
3-(1H-indol-3-yl)maleimide hydrochloride; H-89 (N-[p-
bromocinnamylamino]ethyl)-5-isouquinolinesulfonamide di-
hydrochloride); ionomycin; metaproterenol hemisulfate;
LY294002 (2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-
one hydrochloride); and phenylephrine hydrochloride.
PD98059 (2’-amino-3’-methoxyflavone), platelet-derived
growth factor (PDGF)-BB (human recombinant), phenyl-
ephrine hydrochloride, U-73122 (1-6-[17b-3-methoxy-
extra-1,3,5(10)-trien-17-y]aminohexyl)-1H-pyrrrole-2,5-
dione) and UK14304 (5-bromo-N-(4,5-dihydro-1H-imidazol-
-2-yl)-6-quinolinamine) were obtained from Enzo Life Sci-
ences. (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). Colla-
genase (type II) was obtained from Worthington Biochemical
Co. (Freehold, NJ, U.S.A.). Anti-phospho-p42/p44 MAPK
monoclonal antibody and anti-p42/p44 MAPK monoclonal
antibody, anti-phospho-mammalian target of rapamycin
(mTOR) (Ser2481) polyclonal antibody, anti-mTOR an-
tibody, anti-phospho-mammalian target of rapamycin
(phytch) (Ser2448) polyclonal antibody, and anti-mTOR
antibody were obtained from Cell Signaling Technology (Dan-
vers, MA, U.S.A.). [Methyl-3H]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 ob-
tained from Saitama Experimental Animal Co. (Saitama,
Japan). Adaptation to a light-, humidity- and temperature-
controlled room occurred over a minimum 3-d period prior to
the start of experiments. Rats were fed 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 Technol-
ogy of Japan and the Guidelines for the Care and Use of Lab-
oratory Animals of Josai University.

Hepatocyte Isolation and Culture  Male Wistar rats were
anesthetized by intraperitoneal injection of sodium pen-
tobarbital (45 mg/kg). Two-step in-situ collagenase perfu-
sion was performed in order to facilitate disaggregation of adult
rat livers, as described previously. After perfusion, cells
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-
aminoethyl ether)-N,N',N''-tetraacetic acid (EGTA), 2 mM
dithiothreitol, 1 mM sodium orthovanadate, 1 mM phenyl-
 methylsulphonyl fluoride, 10 μg/ml leupeptin, 10 μg/ml
aprotinin) was added, after which hepatocytes were har-
vested. After centrifugation at 16300×g for 30 min at 4 °C,
cell lysates were denatured in boiling water for 5 min. Sam-
ple of supernatant (30 μg of protein) were subjected to
sodium dodecyl sulfate-polyacrylamide gel electrophoresis
(6% PAGE) using a 10% acrylamide resolving gel, in ac-
cordance with the method of Laemmli. After electrophoresis,
proteins were transferred to Immobilon-P membranes.

For detection of phosphorylated extracellular-regulated protein kinases (pERK1 and pERK2), membranes were im-
mersed in Tris-buffered saline (pH 7.4) containing 1% bovine serum albumin. Membranes were then incubated with
antibody (1 μg/ml) against phospho-ERK and/or ERK, and
were washed as described previously. Antibody binding
was visualized by incubation with a horseradish peroxidase-
conjugated donkey anti-rabbit immunoglobulin G (IgG) sec-
ondary antibody (1:3000 dilution); followed by enhanced
chemiluminescence detection (ECL Kit, Amersham, Little
Chalfont, U.K.). Densitometry 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 using a
modified Lowry procedure with bovine serum albumin as a
standard.

Determination of the Mammalian Target of Rapamycin
(mTOR) Activity  The mTOR protein was identified by im-
munoprecipitation and subsequent immunoblotting with the
corresponding anti-phospho-mTOR 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 cultured in Williams’ medium E con-
taining 5% newborn bovine serum. Cultured hepatocytes were
washed once with ice-cold phosphate-buffered saline
(pH 7.4) and 0.2 ml lysis buffer (10 mM Tris buffer, pH 7.6,
1% Triton X-100, 50 mM NaCl, 5 mM ethylenediamine-
N,N,N',N''-tetraacetic acid (EDTA), 30 mM sodium pyrophos-
phate, 0.01 mM sodium orthovanadate, 50 mM NaF 1 mM
phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 10 μg/ml
aprotinin) was added. Cell lysates were obtained by scraping
the cells in lysis buffer followed by sonication for 1 min. Cell
lysates were then spun down (15,000×g for 10 min at 4 °C),
3.3×10^4 cells/cm^2 in Williams’ medium E containing 5% bovine calf serum, 0.1 mM dexamethasone for 3 h at 37 °C in
5% CO2 in air. The medium was then changed, and cells
were cultured in serum- and dexamethasone-free Williams’
medium E containing PDGF (10 ng/ml) with or without α1-,
α2- and β2-adrenergic agonists and/or specific effectors or in-
hibitors of signal transducers for the indicated times at 37 °C.

Measurement of Extracellular-Signal Regulated Kinase
(ERK) Activity  Phosphorylated ERK isoforms (ERK2;
p42 MAPK, and ERK1; p44 MAPK) were identified by
Western blotting analysis using anti-phospho-ERKs (pERK1
and pERK2) monoclonal antibody as described by Towbin et
al. Phosphorylated ERK activity was normalized against
total ERKs (ERK1 and ERK2). 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-
aminoethyl ether)-N,N',N''-N''-tetraacetic acid (EGTA), 2 mM
dithiothreitol, 1 mM sodium orthovanadate, 1 mM phenyl-
methylsulphinyl fluoride, 10 μg/ml leupeptin, 10 μg/ml
aprotinin) was added, after which hepatocytes were har-
vested. After centrifugation at 16300×g for 30 min at 4 °C,
cell lysates were denatured in boiling water for 5 min. Sam-
ples of supernatant (30 μg of protein) were subjected to
sodium dodecyl sulfate-polyacrylamide gel electrophoresis
(6% PAGE) using a 10% acrylamide resolving gel, in ac-
cordance with the method of Laemmli. After electrophoresis,
proteins were transferred to Immobilon-P membranes.

For detection of phosphorylated extracellular-regulated protein kinases (pERK1 and pERK2), membranes were im-
mersed in Tris-buffered saline (pH 7.4) containing 1% bovine serum albumin. Membranes were then incubated with
antibody (1 μg/ml) against phospho-ERK and/or ERK, and
were washed as described previously. Antibody binding
was visualized by incubation with a horseradish peroxidase-
conjugated donkey anti-rabbit immunoglobulin G (IgG) sec-
ondary antibody (1:3000 dilution); followed by enhanced
chemiluminescence detection (ECL Kit, Amersham, Little
Chalfont, U.K.). Densitometry 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.
and the supernatants were denatured in boiling water for 5 min. For immunoblotting analysis using anti-phospho-
mTOR (Ser2481) polyclonal antibody, samples of the super-
natant (30 μg/lane) were resolved by SDS-PAGE (7.5% poly-
acrylamide gel), transferred to polyvinylidene difluoride
(PVDF) membrane, and immunoblotted with anti-phospho-
tyrosine antibodies. Blots were developed using enhanced
chemiluminescence following incubation with horseradish
peroxidase-conjugated secondary antibodies.22) The super-
natant protein concentration was determined using Lowry’s
assay method.23)

Quantification was performed by densitometry after de-
velopment of the membrane with the enhanced chemilumines-
cence 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 au-
todiagram is representative of three experiments using differ-
ent cell preparations.

Measurement of Hepatocyte DNA Synthesis and Prolif-
eration Hepatocyte DNA synthesis was assessed by meas-
uring by [3H]-thymidine incorporation into acid-precipitable
materials.25) After an initial attachment period of 3 h, the he-
patocytes were washed twice with serum-free Williams’
medium E and cultured in a medium containing PDGF
(10 ng/ml) for a further 4 h. The cells were pulsed at 2 h
and post-PDGF-stimulation for 2 h with [3H]-thymidine (1.0
μCi/well). Incorporation of [3H]-thymidine into DNA was
determined as described previously.11) Hepatocyte protein
content was determined by a modified Lowry procedure,23)
using bovine serum albumin as the standard. Data are ex-
pressed 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.11,16)

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

RESULTS

Time Course and Patterns of ERK Isoform Activity
Stimulated by PDGF It has been reported that ERK plays
an important role in the proliferation of hepatocytes and
other cells induced by growth factors and cytokines.26,27) The
typical detectable pattern of phospho-ERK on Western blot-
ing analysis is shown in Fig. 1A. 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
10 ng/ml PDGF, a concentration that stimulated hepatocyte
proliferation (Fig. 1B), and the band returned to basal levels
within 60 min (Fig. 1B). ERK1 was not significantly affected
by either medium alone (control) or 10 ng/ml PDGF treat-
ment (Fig. 1C).

Effects of Specific Inhibitors of Signal Transducers on
PDGF-Stimulated ERK Isoform Activity In order to charac-
terize the involvement of ERK in the mitogenic path-
way induced by PDGF, we investigated the effects of the
PDGF receptor tyrosine kinase inhibitor AG1296 (10−7 M28),
the phospholipase C (PLC) inhibitor U-73122 (10−6 M29), the
PI3 kinase inhibitor LY294002 (10−7 M30), the MEK in-
hibitor PD98059 (10−8 M31), the mammalian target of ra-
panycin (mTOR) inhibitor rapamycin (10 ng/ml32) and the
PKC inhibitor GF109203X (10−5 M33) on PDGF-induced
ERK activity. As shown in Fig. 2, the phosphorylation of
ERK2 induced by PDGF (10 ng/ml) at 3 min was almost
completely blocked by AG1296, U-73122, LY294002 and
PD98059. However, rapamycin and GF109203X did not af-
flect the PDGF-induced ERK2 activity. In addition, phos-
phorylation of ERK1 in the presence of 10 ng/ml PDGF was not
affected by these inhibitors.
Activation of ERK2 Produced by PDGF Is Potentiated by α1-Adrenergic Agonist Phenylephrine  The potentiating effects of α1-adrenergic agonists on the induction of ERK pathway by 10 ng/ml PDGF were investigated using the α1-adrenergic agonist phenylephrine. PDGF in the presence of phenylephrine (10^{-6} M) caused a more rapid and significant increase in ERK2 activity than PDGF alone. ERK2 activity peaked at 3 min (about 3.0-fold increase from baseline) after addition and rapidly decreased to basal levels within 60 min (Figs. 3A, B). In contrast, phenylephrine alone did not significantly stimulate ERK2 activity. Phenylephrine (10^{-6} M) in the presence of 10 ng/ml PDGF did not significantly stimulate ERK2 activity. Phenylephrine (10^{-6} M) is known to have the ability to potentiate PDGF-induced ERK2 activity.

Effects of Specific Inhibitors of Signal Transducers on α1-Adrenergic Agonist- and/or TPA-Induced ERK Isoform Activity in the Presence of PDGF  In order to investigate the potentitating mechanism for the α1-adrenergic receptor/protein kinase C (PKC) pathway in induction of ERK2 activity by PDGF, we examined the effects of phenylephrine (indirect PKC activator), 12-O-tetradecanoylphorbol-13-acetate, (phorbol ester, TPA; direct PKC activator) and ionomycin (ionophore of the intracellular Ca^{2+}) on the phosphorylation of ERK isoforms induced by 10 ng/ml PDGF. Both phenylephrine (10^{-6} M) and TPA (10^{-7} M)-induced potentiation of ERK2 phosphorylation in the presence of PDGF were almost completely blocked by AG1296, U-73122, LY294002 and PD98059 (Fig. 4). However, they were not blocked by rapamycin. In contrast, pretreatment of hepatocytes with the PKC inhibitor GF109203X (10^{-7} M) blocked the potentiation effects of phenylephrine and TPA on the phosphorylation of ERK2 induced by PDGF. Moreover, ionomycin (10^{-5} M), a Ca^{2+}-transporting ionophore that raises intracellular levels of Ca^{2+} in the cytosol from across biological membranes, did not potentiate ERK2 phosphorylation by PDGF. Phenylephrine (10^{-6} M) or TPA (10^{-7} M) alone had no significant effect on the phosphorylation of ERK1 and ERK2 (Fig. 4).

Effects of Specific Inhibitors of Signal Transducers on UK14304, Metaproterenol and/or 8-Bromo cAMP-Induced ERK Isoform Activity in the Presence of PDGF  Hepatocytes were stimulated with PDGF (10 ng/ml) plus UK14304 (10^{-6} M), metaproterenol (10^{-6} M) and/or 8-bromo cAMP (10^{-6} M) in the presence of specific signal transducer inhibitors such as AG1296 (10^{-7} M), LY294002 (10^{-7} M), PD98059 (10^{-6} M), rapamycin (10 ng/ml) and H-89 (10^{-7} M). Phosphorylated ERK isoforms (pERK1 and pERK2) were determined after incubation for 3min with test agents. Phosphorylated ERK isoforms (pERK1 and pERK2) were identified using anti-phospho-ERK1/2 (p42/44 MAPK) antibody, as described in Materials and Methods. Results are expressed as a percentage of the respective control values (mean±S.E.M. of three experiments). *p<0.05; **p<0.01 vs. respective controls (PDGF alone).

Fig. 3. Effects of Phenylephrine on Time Course and Patterns of ERK Isoform Activity Induced by PDGF  Hepatocytes were stimulated with PDGF (10 ng/ml) in the absence or presence of phenylephrine (10^{-6} M). Phosphorylated ERK (44 kDa) typical Western blotting band (B) pERK2, and (C) pERK1 were identified using anti-phospho-ERK1/2 (p42/44 MAPK) antibody and/or anti-ERK, as described in Materials and Methods. Results are expressed as a percentage of the respective control values (mean±S.E.M. of three experiments). *p<0.05; ++p<0.01 vs. respective controls. C: control; P: PDGF; Phen: phenylephrine.

Fig. 4. Effects of Specific Inhibitors of Signal Transducers on Phenylephrine and/or TPA-Induced ERK Isoform Activity in the Presence of PDGF  Hepatocytes were stimulated with PDGF (10 ng/ml) plus phenylephrine (10^{-6} M) and/or TPA (10^{-7} M) in the presence of specific inhibitors of signal transducers, such as AG1296 (10^{-7} M), LY294002 (10^{-7} M), PD98059 (10^{-6} M), rapamycin (10 ng/ml), U-73122 (10^{-5} M), GF109203X (10^{-5} M) and ionomycin (10^{-5} M). Phosphorylated ERK isoforms (pERK1 and pERK2) were determined after incubation for 3 min with test agents. Phosphorylated ERK isoforms (pERK1 and pERK2) were identified using anti-phospho-ERK1/2 (p42/44 MAPK) antibody, as described in Materials and Methods. Results are expressed as a percentage of the respective control values (mean±S.E.M. of three experiments). *p<0.05; **p<0.01 vs. respective controls (PDGF alone).

Fig. 5. Effects of Specific Inhibitors of Signal Transducers on Metaproterenol and/or 8-Bromo cAMP-Induced ERK Isoform Activity in the Presence of PDGF  Hepatocytes were stimulated with PDGF (10 ng/ml) plus UK14304 (10^{-6} M), metaproterenol (10^{-6} M) and/or 8-bromo cAMP (10^{-6} M) in the presence of specific signal transducer inhibitors such as AG1296 (10^{-7} M), LY294002 (10^{-7} M), PD98059 (10^{-6} M), rapamycin (10 ng/ml) and H-89 (10^{-7} M). Phosphorylated ERK isoforms (pERK1 and pERK2) were determined after incubation for 3 min with the test agents. Phosphorylated ERK isoforms (pERK1 and pERK2) were identified using anti-phospho-ERK1/2 (p42/44 MAPK) antibody, as described in Materials and Methods. Results are expressed as a percentage of the respective control values (mean±S.E.M. of three experiments). *p<0.05; **p<0.01 vs. respective controls (PDGF alone).
...and/or 8-bromo cAMP (10^{-7} M; direct PKA stimulator)\(^{37}\), significant potentiation of ERK2 activity was not observed when compared with PDGF (10 ng/ml) alone (Fig. 5). PDGF-induced ERK2 phosphorylation in the presence of metaproterenol and 8-bromo-cAMP was also blocked by AG1296, LY294002 and PD98059, but was not blocked by rapamycin. In addition, pretreatment of hepatocytes with the PKA inhibitor H-89 (10^{-7} M) did not block the phosphorylation of ERK2 in the presence of PDGF. UK14304 (10^{-7} M), metaproterenol (10^{-6} M), 8-bromo cAMP (10^{-7} M) and H-89 (10^{-7} M) alone had no significant effect on the phosphorylation of ERK1 and ERK2 (Fig. 5).

**Effect of Specific Inhibitors of Growth-Related Signal Transducers and on TPA-Induced Mammalian Target of Rapamycin (mTOR) Activity in the Presence of PDGF**

To obtain further support for the cross-talk between PDGF receptor mediated-ERK2-induced mTOR activation (mTOR phosphorylation), and \(\alpha_1\)-adrenergic receptor activities, we examined the effects of specific inhibitors of growth-related signal transducers on PDGF-induced mTOR phosphorylation by \(\alpha_1\)-receptor agonist phenylephrine and TPA. Figure 6 shows that PDGF (10 ng/ml) caused an increase in the mTOR phosphorylation of a 289-kDa protein that peaked 5—10 min after addition, at about 2.2-fold (compared with control). When PDGF (10 ng/ml) was added in combination with AG1296 (10^{-7} M), LY294002 (10^{-6} M), PD98059 (10^{-6} M), rapamycin (10 ng/ml), U-73122 (10^{-7} M), and GF109203X (10^{-7} M), respectively, AG1296, LY294002, PD98059, rapamycin, and U-73122 completely abolished the PDGF-induced increase in mTOR phosphorylation (Ser 2481). In contrast, PDGF-induced mTOR activation was not abolished by GF109203X (10^{-7} M) treatment. In addition, both \(\alpha_1\)-adrenergic receptor agonist phenylephrine (10^{-6} M) and direct PKC activator TPA (10^{-7} M) increased in PDGF-induced the mTOR phosphorylation at about 4.5-fold (compared with control). The TPA (10^{-7} M)-induced potentiation of mTOR phosphorylation in the presence of PDGF was almost completely blocked by AG1296, U-73122, LY294002, PD98059 and rapamycin (Fig. 6). However, pretreatment of hepatocytes with the PKC inhibitor GF109203X (10^{-7} M) blocked the potentiative effects of TPA on the phosphorylation of mTOR induced by PDGF. Phenylephrine (10^{-6} M) or TPA (10^{-7} M) alone had no significant effect on the phosphorylation of mTOR (Fig. 6).

**Effects of Specific Inhibitors of Growth-Related Signal Transducers on Hepatocyte DNA Synthesis and Proliferation Induced by PDGF**

In order to confirm the notion that 10 ng/ml PDGF induces hepatocyte DNA synthesis and proliferation through PDGF receptor, ERK or mTOR phosphorylation, we investigated whether or not AG1296, LY294002, PD98059, and rapamycin are able to inhibit PDGF-induced hepatocyte DNA synthesis and proliferation in the presence or absence of phenylephrine or TPA. As shown in Fig. 7, AG1296 (10^{-7} M), LY294002 (10^{-6} M), PD98059 (10^{-6} M), rapamycin (10 ng/ml) and U-73122 (10^{-7} M) almost completely blocked PDGF-induced hepatocyte DNA synthesis and proliferation with or without phenylephrine (10^{-6} M) or TPA (10^{-7} M). In addition, the potentiating effects of phenylephrine (10^{-6} M) or TPA (10^{-7} M) on PDGF-induced hepatocyte DNA synthesis and proliferation was blocked by GF109203X (10^{-7} M) treatment. Phenylephrine (10^{-6} M) or TPA (10^{-7} M) alone had no any effect on PDGF-induced DNA synthesis and proliferation (Fig. 7).

**DISCUSSION**

The proliferative pathway by which PDGF activates the re-ceptor tyrosine kinase/ERK cascade (ERK pathway) has been described in many normal cells and transformed cells.\(^{4,5,13—15}\) However, there have been few studies regarding the PDGF-induced activation of ERK isoforms and cross-
talk with adrenergic signal transduction pathways in primary cultures of adult rat hepatocytes. Therefore, we investigated the possible roles of ERK isoform activity and other signal transducers induced by mitogenic dose of PDGF in the absence or presence of adrenergic receptor agonists.

As shown in Figs. 1 and 2, we demonstrated that PDGF rapidly stimulates ERK2 activity, but not ERK1 activity. Cárcamo-Orive et al., show that continued expression of cyclin-D1 in the G1/S phase is dependent on ERK2 expression by PDGF, and that the ablation of ERK1 influences neither the proliferation capacity of human mesenchymal stem cells, nor the expression pattern of cyclin-D1. In agreement with a recent report, our data are suggested that ERK2 play a key role in hepatocyte proliferation. The ERK2 activity was blocked by the MEK inhibitor PD98059. Moreover, hepatocyte ERK2 activity induced by PDGF was almost completely blocked by specific inhibitors of growth-related signal transducers, such as AG1296, U-73122 and LY294002, but not rapamycin or GF109203X (Fig. 2). These results suggest that signal transducers such as receptor tyrosine kinase of PDGF, PLC, ERK2, PI3K and mTOR play an essential role in the mitogenic activity induced by PDGF under these experimental conditions (Fig. 6). The present results are consistent with reports that ERK2 acts upstream of mTOR/p70S6K and downstream of receptor tyrosine kinase, PLC and PI3K in primary cultures of adult rat hepatocytes and cancer cell lines. In addition, GF109203X-sensitive PKC is not involved in the PDGF signaling pathway.

There have been very few studies on the adrenergic agonist-induced regulation of ERK activity. Thus, we examined whether ERK2 activity, and β2-adrenergic agonists modulate the PDGF-induced changes in the activity of each ERK isoform. Phenylephrine is known to exert its action through the stimulation of phospholipase C (PLC-β) via α2-adrenergic receptor/Gq-protein. PLC-β catalyzes the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP2) to inositol 1,4,5-triphosphate (IP3) and diacylglycerol (DG). Generation of IP3 leads to the mobilization of calcium ions from intracellular stores, whereas DG activates different isoforms of the PKC family.

As shown in Fig. 3, PDGF-induced hepatocyte ERK2 activity was enhanced by phenylephrine (10⁻⁷ M), an α2-adrenergic receptor agonist and an indirect PKC activator (Figs. 3, 4). In contrast, phenylephrine alone had no significant effect on hepatocyte ERK activity in the absence of PDGF (Fig. 4). The enhancing effects of phenylephrine in the presence of PDGF were inhibited by both the PLC inhibitor U-73122 and the PKC inhibitor GF109203X (10⁻⁷ M), thus suggesting the involvement of PLC and PKC in the potentiating effects of phenylephrine (Fig. 4). In support of this notion, phorbol ester, 12-O-tetradecanoylphorbol-13-acetate (TPA; 10⁻⁷ M), is a direct activator of PKC, and can either directly or indirectly stimulate PDGF-induced ERK2 phosphorylation. The α2-adrenergic receptor-mediated signals may interact with upstream signal transducers, such as the MEK, Raf or ras pathways.

However, as the specific PKC inhibitor GF109203X alone had no significant effect on PDGF-induced ERK2 phosphorylation (Fig. 2), GF109203X-sensitive PKC does not appear to be involved in the PDGF-induced ERK2 phosphorylation. Taken together, these results suggest that PLC and PKC are involved in the PDGF pathway, but these enzyme subtypes are apparently different from one another. Accordingly, it has been reported that some PLC-γ-mediated cellular responses have been shown to be independent of the PLC-β-mediated pathway. PLC-γ-β, but not PLC-β may be activated by the phosphorylation of growth factor receptor tyrosine kinase via the Src homology (SH) domain.

Accordingly, PDGF-induced hepatocyte DNA synthesis and proliferation were almost completely blocked by AG1296, LY294002, PD98059, U-73122 and rapamycin (Fig. 7). Moreover, the involvement of PKC in the effects of phenylephrine is supported by the present results, indicating that phenylephrine- or TPA-induced potentiation of hepatocyte DNA synthesis and proliferation in the presence of PDGF (10 ng/ml) was completely inhibited by the PKC inhibitor GF109203X (10⁻⁷ M) treatment (Fig. 7). These results support the notion that ERK2 play a key role in cross-talk between PDGF receptor/PLC-γ/ERK and α2-adrenergic receptor/PLC-β/PKC pathway (Fig. 8). On the other hand, the PDGF-induced hepatocyte ERK2 activity was not enhanced by a maximal dose of U.K.14304 (10⁻⁶ M), α2-adrenergic receptor agonist (indirect adenylate cyclase inhibitor) and/or metaproterenol (10⁻⁸ M), a β2-adrenergic receptor agonist (indirect adenylate cyclase activator) or the cell-permeable cAMP analog, 8-bromo cAMP (10⁻⁷ M, Fig. 5). These results indicate that the α2- or β2-adrenergic receptor/cAMP pathway was not involved in cross-talk with the PDGF receptor/ERK pathway to potentiate hepatocyte growth.

A possible scheme for cross-talk between the PDGF receptor/PLC-γ/ERK pathway and the α2-adrenergic receptor/PLC-β/PKC pathway is shown in Fig. 8. However, more detailed mechanisms for cross-talk between the PDGF-signalizing pathway and the α2-adrenergic receptor/PLC/PKC pathway remain to be explored.

In conclusion, the present study demonstrates that the cross-talk signals by extracellular α2-adrenergic receptor agonists, such as phenylephrine, potentiate the PDGF-induced ERK2 activity in primary cultured adult rat hepatocytes, and that...
the cross-talk element may be present downstream of PDGF receptor tyrosine kinase, and upstream of MEK (i.e., ras, Raf and fos), PI3K, mTOR and p70 S6K (Fig. 8). The present findings support the notion that endogenous catecholamine-induced potentiation of hepatocyte DNA synthesis and proliferation in the presence of PDGF is mediated by \(\alpha_2\)-adrenergic receptors and plays an important role in the potentiation of the ERK cascade during liver regeneration after partial hepatectomy or liver necrosis caused by toxic chemicals in vivo.\(^{6—8}\)

**Acknowledgement** This research was partially supported by a Grant-in-Aid for Exploratory Research (No. 14771291, 2003) from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

**REFERENCES**