0022-3565/97/2823-1146$03.00/0
THE JOURNAL OF PHARMACOLOGY AND EXPERIMENTAL THERAPEUTICS
JPET 282:1146–1154, 1997

Vol. 282, No. 3
Printed in U.S.A.

Proliferation of Adult Rat Hepatocytes by Hepatocyte Growth Factor Is Potentiated by Both Phenylephrine and Metaproterenol

MITSUTOSHI KIMURA and MASAHIKO OGIHARA

Biochemical Pharmacology Group, Faculty of Pharmaceutical Sciences, Josai University. 1–1, Keyakidai, Sakado, Saitama 350–02 Japan

Accepted for publication May 1, 1997

ABSTRACT

We investigated whether or not *beta* and *alpha* adrenergic agonists could affect proliferation of adult rat hepatocytes induced by hepatocyte growth factor (HGF) during the early and late phases of primary culture. Adult rat hepatocytes underwent significant DNA synthesis after culture with 5 ng/ml HGF for 3 h at a low cell density (3.3×10^4 cells/cm^2). Under these culture conditions, the number of nuclei increased significantly during a subsequent 4-h culture period. Hepatocyte DNA synthesis and proliferation induced by 5 ng/ml HGF was reduced at high cell densities near confluence. A *beta* adrenergic agonist, metaproterenol (10^{-7} M), and dibutyryl cAMP significantly potentiated hepatocyte DNA synthesis and proliferation at a concentration as low as 10^{-7} M when cultured in combination with 5 ng/ml HGF. Similarly, an *alpha*-1 adrenergic agonist, phenylephrine (10^{-6}–10^{-4} M) markedly potentiated HGF-induced hepatocyte DNA synthesis and proliferation. The phenylephrine effect was mimicked by a phorbol ester (10^{-6} M), but not by ionomycin (10^{-6} M). The mitogenic effects of HGF were almost completely blocked by simultaneous treatment of hepatocytes with genistein (5×10^{-6} M), U-73122 (10^{-6} M), wortmannin (10^{-7} M), sphingosine (3×10^{-6} M) and rapamycin (10 ng/ml). These results demonstrate that HGF can rapidly induce proliferation of adult rat hepatocytes in primary culture. However, this effect is dependent on the initial plating density. The comitogenic effects of metaproterenol and phenylephrine may involve both protein kinase A and protein kinase C activation, respectively. The results also suggest that following stimulation with HGF, activation of tyrosine kinase, phosphatidylinositol 3-kinase, phospholipase C and p70 ribosomal protein S6 kinase is essential for hepatocyte proliferation.

Liver regeneration in response to partial hepatectomy or chemical liver injury is a physiological growth response observed in intact animals (Sandnes *et al.*, 1986; Michalopoulos, 1990). During liver regeneration, quiescent hepatocytes undergo one or two rounds of replication and then return to a nonproliferative state. Growth factors regulate this process by providing both stimulatory and inhibitory signals for cell proliferation. A variety of growth factors, including EGF and HGF, have potent mitogenic effects on hepatocytes and stimulate normal liver growth and liver regeneration (Nakamura *et al.*, 1983b). HGF is a potent mitogen first purified from rat platelet and human and rabbit plasma (Nakamura *et al.*, 1986, 1987, 1989). The response of adult rat hepatocytes to HGF and other growth factors has been studied extensively with respect to DNA synthesis and proliferation *in vitro* (Richman *et al.*, 1976; McGowan *et al.*, 1981; Nakamura *et al.*, 1983a; Marker *et al.*, 1992). However, such experiments were performed during the relatively late phases of culture (*i.e.,* 24– 48 h).

We have reported previously that EGF and insulin alone can rapidly stimulate hepatocyte DNA synthesis and proliferation during short-term (*i.e.,* approximately 4 h) cultures (Kimura and Ogihara, 1997a; Kimura and Ogihara, in press, 1997b). Depending on the growth factor, hepatocyte proliferation is dependent on the plating density. For example, hepatocyte DNA synthesis and proliferation induced by EGF is strictly dependent on the initial plating density, whereas that induced by insulin does not depend exclusively on initial plating density. Furthermore, hepatocyte proliferation appears to be potentiated by *beta* adrenergic agonists and other cAMP-elevating agents.

Recently, the signal transduction pathway activated in

Received for publication February 12, 1997.

ABBREVIATIONS: HGF, hepatocyte growth factor; EGF, epidermal growth factor; DNA, deoxyribonucleic acid; PI(3)K, phosphatidylinositol 3-kinase; p70 S6K, P70 ribosomal protein S6 kinase; cAMP, adenosine 3′,5′-cyclic monophosphate; UK-14304, 5-bromo-6-[2-imidazolin-2-ylamino]-quinoxaline; U-73122, (1-[-[[17β-3-methoxyestra-1, 3, 5 (10)-triene-17-yl] amino] hexyl]-1H pyrrol-2, 5-dione); db-cAMP, N^6,2′-o-dibutyryl cAMP; H-89, N-[2-(p-bromocinnamylamino) ethyl]-5-isoquinolinesulfonamide; PKA, protein kinase A; PKC, protein kinase C; PLC, phospholipase C; DG, 1, 2-diacylglycerol; IP_3, inositol 1,4,5-trisphosphate; PMA, phorbol myristate acetate; PDGF, platelet-derived growth factor; HEPES, N-[2-hydroxy-ethyl]-piperazine-N′-[2-ethane sulfonic acid].

response to HGF in hepatocytes has become understood more clearly (Marker *et al.*, 1992; Gines *et al.*, 1995). HGF initiates its proliferative effects through the activation of tyrosine kinase-linked receptors and can induce replication in adult rat hepatocytes (Osada *et al.*, 1992). However, the precise mechanism by which HGF acts remains unclear. Thus, the present study investigated the possibility that HGF alone also participates in the intracellular events involved in rapid proliferation of adult rat hepatocytes. In addition, we examined the effects of *alpha* and *beta* adrenergic agonists on HGF-induced DNA synthesis in adult rat hepatocytes to clarify the relationship between HGF action and the adrenergic responses. Finally, we investigated pharmacologically the cell signaling systems involved in the HGF responsiveness in primary cultures of adult rat hepatocytes.

Materials and Methods

Hepatocyte isolation and culture. Male Wistar rats (weight 200–250 g) were obtained from Saitama Experimental Co. (Saitama, Japan). The rats were anesthetized by intraperitoneal injection of sodium pentobarbital (45 mg/kg). A two-step *in situ* collagenase perfusion was performed to facilitate disaggregation of the adult rat liver as described previously (Seglen, 1975; Ogihara, 1995). The liver was first washed *via* the portal vein with Ca^{++}-free Hanks-10 mM HEPES buffer (pH 7.4) at 37°C and a flow rate of 30 ml/min for 10 min. The second step was performed with use of the same buffer containing 0.025% collagenase and 0.075% $CaCl_2$ at a flow rate of 30 ml/min for 10 min. The cells were dispersed in Ca^{++}-free Hanks' solution. The cells were then washed three times by slow centrifugation ($120 \times g$) for 1 min to remove cell debris, damaged cells and nonparenchymal cells. The viability of hepatocytes was monitored by trypan blue dye exclusion. On average, more than 94% of the cells remained intact. Unless otherwise indicated, isolated hepatocytes were plated onto plastic culture dishes (Sumitomo Bakelite Co., Tokyo, Japan) at a density of 3.3×10^4 cells/cm^2 in Williams' medium E containing 5% bovine calf serum, 10^{-10} M dexamethasone for 3 h 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 various concentrations of HGF with or without *beta* adrenergic agonists, cAMP-elevating agents, an *alpha*-1 adrenergic agonist and/or specific inhibitors of signal transducers.

Measurement of DNA synthesis. Hepatocyte DNA synthesis was assessed by measuring [^{3}H]thymidine incorporation into acid-precipitable materials (Morley and Kingdon, 1972). 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 5 ng/ml HGF for a further 4 h and 21 h. The cells were pulsed at 2 h and 19 h post-HGF stimulation for 2 h with [^{3}H]thymidine (1.0 μCi/well). Incorporation into DNA was determined as described previously (Kimura and Ogihara, 1997a). The hepatocyte protein content was measured by a modified Lowry procedure with bovine serum albumin as a standard (Lee and Paxman, 1972).

Counting nuclei. The number of nuclei was counted instead of the cell number according to the previously described procedure of Nakamura *et al.* (1983a) with minor modifications. The cultured hepatocytes were washed twice with 2 ml of Dulbecco's phosphate-buffered saline (pH 7.4). Then, the cells were lysed by incubation with 0.25 ml of 0.1 M citric acid containing 0.1% Triton X-100 for 30 min at 37°C. An equal volume of the nucleus suspension was mixed with 0.3% trypan blue in Dulbecco's phosphate-buffered saline and the number of nuclei was counted in a hemocytometer. This procedure was performed because the hepatocytes firmly attached to the collagen-coated plates and were not dispersed by EDTA-trypsin treatment.

Materials. The following reagents were obtained from Sigma Chemical Co. (St. Louis, MO): HGF (human recombinant), forskolin, db-cAMP, genistein, forskolin, aphidicolin, metaproterenol hemisulfate, butoxamine hydrochloride, metoprolol tartrate, dobutamine hydrochloride, phenylephrine hydrochloride, D-sphingosine, ionomycin calcium salt, UK14304, glucagon (porcine), wortmannin, rapamycin and dexamethasone. H-892HCl and U-73122 were obtained from BIOMOL, Research Laboratories Inc. (Plymouth Meeting, PA). PMA was purchased from Research Biochemicals International (Natick, MA). 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). [methyl-^{3}H]Thymidine (20 Ci/mmol) was obtained from DuPont-New England Nuclear (Boston, MA). All reagents were of analytical grade.

Statistical analysis. Values are expressed as mean ± S.E.M. Data were analyzed by the unpaired Student's *t* test. P values less than 0.05 were regarded as statistically significant.

Results

Time course associated with stimulation of hepatocyte DNA synthesis and proliferation induced by HGF with or without metaproterenol. Isolated adult rat hepatocytes were treated with HGF (5 ng/ml) with or without metaproterenol (10^{-7} M) at various points during the culture period, and DNA synthesis was measured by [^{3}H]thymidine incorporation at a low cell density (3.3×10^4 cells/cm^2). DNA synthesis was induced in hepatocytes after only 2 h and reached a maximum 3 h after adding HGF (5 ng/ml). However, DNA synthesis became markedly reduced at 21 h (fig. 1). Hepatocyte DNA synthesis induced by HGF was potentiated in the presence of a *beta*-2 adrenergic agonist, metaproterenol (10^{-7} M) and a nonspecific *beta* adrenergic agonist, isoproterenol (10^{-7} M, not shown), during the early phase of culture. The HGF (5 ng/ml)-induced increase in the number of nuclei (proliferation) began approximately 3.5 h after the addition of HGF and gradually increased for a further 17 h. Proliferation was potentiated by metaproterenol treatment. Therefore, the detected increase in the number of nuclei could be caused by an increase in the [^{3}H]thymidine incorporation after HGF treatment.

Time course associated with stimulation of hepatocyte DNA synthesis and proliferation induced by HGF with or without phenylephrine. Isolated adult rat hepatocytes were treated with HGF (5 ng/ml) with or without phenylephrine (10^{-6} M) at various points during the culture

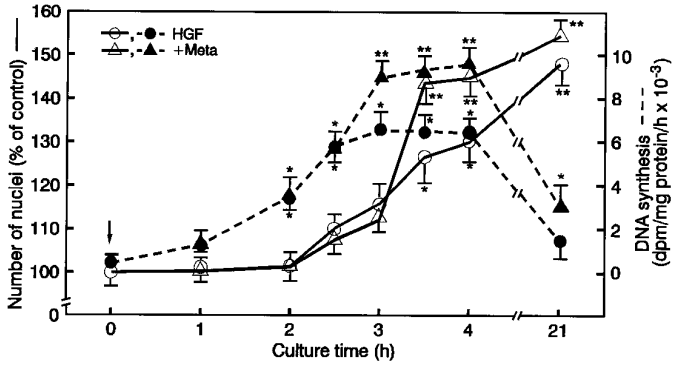

Fig. 1. Time course of stimulation of hepatocyte DNA synthesis and proliferation by HGF with or without metaproterenol (Meta). Hepatocytes (3.3×10^4 cells/cm^2) were cultured with or without metaproterenol (10^{-7} M) in the presence of HGF (5 ng/ml) for various lengths of time. Data are expressed as the mean ± S.E. of three separate experiments. *P < .05, **P < .01 compared with controls (values just before HGF addition).

period, and DNA synthesis was measured by [^{3}H]thymidine incorporation at a low cell density (3.3×10^4 cells/cm^2). DNA synthesis was induced in hepatocytes after only 2.5 h and reached a maximum 3 to 4 h after the addition of HGF (5 ng/ml). Proliferation became markedly reduced by 21 h (fig. 2). Hepatocyte DNA synthesis induced by HGF was potentiated in the presence of an *alpha*-1 adrenergic agonist, phenylephrine (10^{-6} M) during early phase of culture. The HGF (5 ng/ml)-induced increase in the number of nuclei (proliferation) began approximately 3.5 h after the addition of HGF and gradually increased for a further 17 h. Proliferation was potentiated by phenylephrine treatment.

Effect of dexamethasone pretreatment on HGF-induced hepatocyte DNA synthesis and proliferation during early and late phases of culture. To investigate the mechanism by which HGF rapidly stimulates hepatocyte DNA synthesis and proliferation, we examined the effects of dexamethasone pretreatment (3 h after plating) on HGF-stimulated hepatocyte DNA synthesis and proliferation during the early and late phases of culture. Figure 3A shows that HGF-induced hepatocyte DNA synthesis and proliferation were greatly impaired at the stage of 4-h culture when relatively large doses of dexamethasone (10^{-8} and 10^{-7} M) were added during the 3-h attachment period. The inhibitory effects of dexamethasone on HGF-stimulated hepatocyte DNA synthesis and proliferation (IC_{50} 2.7 ± 0.3 nM) were partially restored to the control level after culture with HGF for 21 h (fig. 3B).

Dose-dependent effect of HGF on hepatocyte DNA synthesis and proliferation. Dose-response effects of HGF on hepatocyte DNA synthesis and proliferation in the low-density culture (3.3×10^4 cells/cm^2) for 4 h were examined. As shown in figure 4, the effect of HGF on hepatocyte DNA synthesis was dose-dependent. Peak stimulation of hepatocyte DNA synthesis was seen at the dose of 3 ng/ml and showed an EC_{50} of 0.95 ± 0.09 ng/ml. The number of nuclei increased dose-dependently by approximately 1.3-fold with HGF administration. The maximal effect of stimulation occurred at approximately 5 ng/ml and showed an EC_{50} of 0.93 ± 0.01 ng/ml (fig. 3).

Influence of cell density on HGF-stimulated hepatocyte DNA synthesis and proliferation with or without metaproterenol and phenylephrine. To study whether or not the proliferative effect of HGF is affected by the initial plating density, we investigated the density dependence of hepatocyte DNA synthesis and proliferation induced by 5 ng/ml HGF with or without metaproterenol or phenylephrine. Figure 5 shows that initial plating density appears to influence an important step involved in hepatocyte DNA synthesis. Hepatocyte DNA synthesis was induced by HGF at low densities, but became markedly reduced at a high cell density that approaches confluence, both in the presence and absence of metaproterenol or phenylephrine. As shown in figure 6, the HGF (5 ng/ml)-induced increase in the number

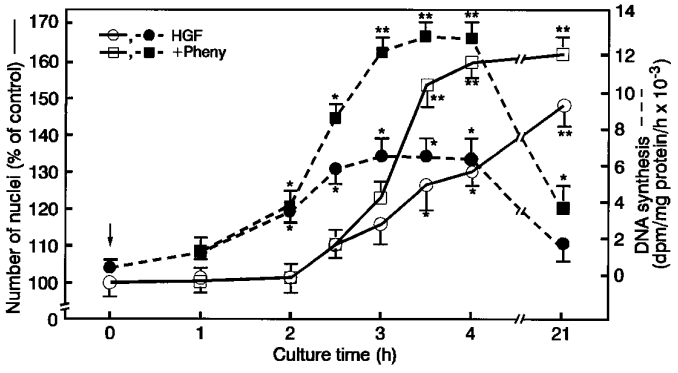

Fig. 2. Time course of stimulation of hepatocyte DNA synthesis and proliferation by HGF with or without phenylephrine (Pheny). Hepatocytes (3.3×10^4 cells/cm^2) were cultured with or without phenylephrine (10^{-6} M) in the presence of HGF (5 ng/ml) for various lengths of time. Data are expressed as mean ± S.E. of three separate experiments. *P < .05, **P < .01 compared with controls (values just before HGF addition).

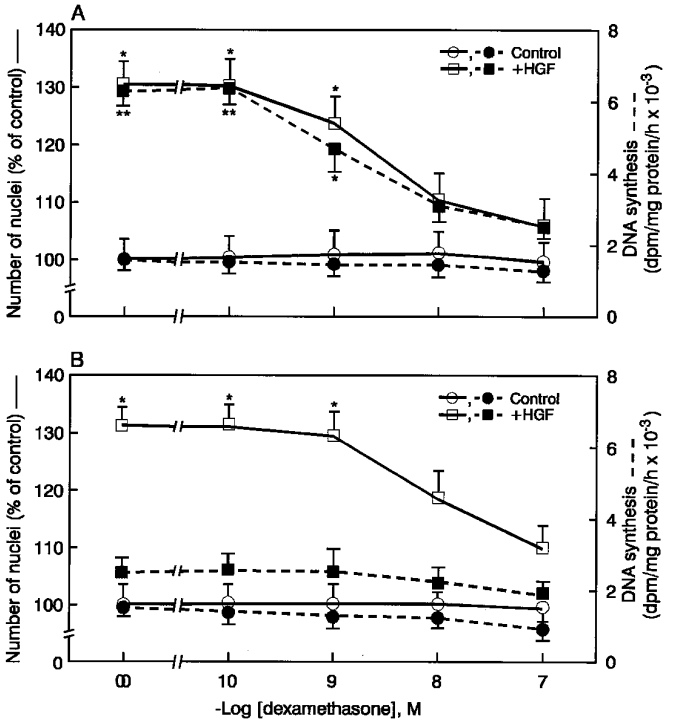

Fig. 3. Effect of dexamethasone pretreatment on HGF-induced hepatocyte DNA synthesis and proliferation during early and late phases of culture. Hepatocytes (3.3×10^4 cells/cm^2) were cultured with or without various concentrations of dexamethasone (10^{-10}–10^{-7} M) in the absence of HGF for 3 h. The medium was then changed, and the cells were cultured in serum- and dexamethsone-free Williams' medium E containing 5 ng/ml HGF. Data are expressed as mean ± S.E. of three separate experiments. *P < .05, **P < .01 compared with respective controls.

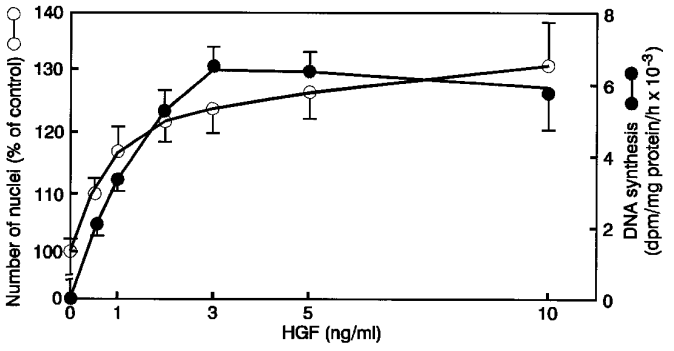

Fig. 4. Dose-dependent effect of HGF on hepatocyte DNA synthesis and proliferation. Hepatocytes (3.3×10^4 cells/cm^2) were cultured with various concentrations of HGF for 4 h. Data are expressed as mean ± S.E. of three separate experiments.

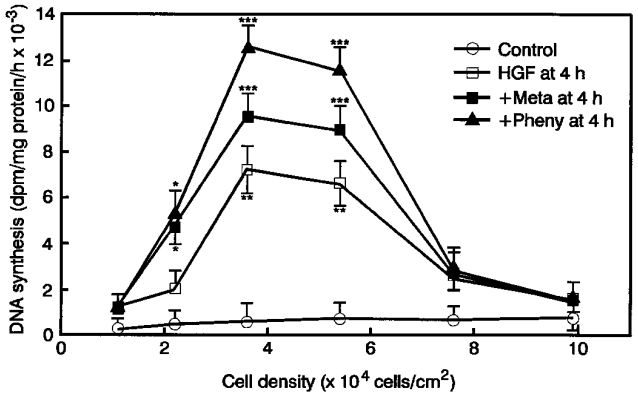

Fig. 5. Influence of cell density on metaproterenol (Meta) (10^{-7} M)- or phenylephrine (Pheny) (10^{-6} M)-stimulated hepatocyte DNA synthesis in presence of HGF. Hepatocytes were cultured with dexamethasone (10^{-10} M) or HGF (5 ng/ml) at various plating densities for 4 h. Data are expressed mean ± S.E. of three separate experiments. *P < .05, **P < .01, ***P < .001 compared with the respective controls.

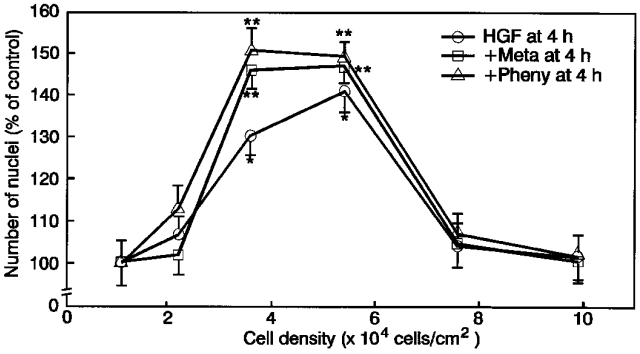

Fig. 6. Influence of cell density on the metaproterenol (Meta) (10^{-7} M)- or phenylephrine (Pheny) (10^{-6} M)-stimulated hepatocyte proliferation in presence of HGF. Hepatocytes were cultured with HGF (5 ng/ml) at various plating densities for 4 h and 21 h. Data are expressed as mean ± S.E. of three separate experiments. *P < .05, **P < .01 compared with respective controls.

of nuclei reached a plateau at a cell density of 3.3×10^4 cells/cm^2. The HGF-induced increase was observed both during the early and late phases of culture (not shown). However, the effects of HGF treatment were reduced or absent at a high cell density, regardless of the presence or absence of metaproterenol or phenylephrine. Hepatocyte DNA synthesis and proliferation in hepatocytes cultured without or with dexamethasone (10^{-10} M) for 21 h did not appear to be affected, regardless of cell density.

Dose-dependent effects of metaproterenol and phenylephrine on HGF-stimulated hepatocyte DNA synthesis and proliferation during the early and late phases of primary culture. To determine the influence of *alpha* and *beta* adrenergic mechanisms on the HGF action, we examined the dose-dependent effects of phenylephrine and metaproterenol on HGF-stimulated DNA synthesis and proliferation at a low density during the early and late phases of culture (table 1). Metaproterenol alone had almost no effect on hepatocyte DNA synthesis and proliferation in the range of 10^{-8} to 10^{-6} M (data not shown). However, the ability of HGF to induce hepatocyte DNA synthesis and proliferation was significantly potentiated by the addition of metaproterenol with the maximal effect seen at a concentration of 10^{-7} M. The potentiation was dose-dependent for metaproterenol up to 10^{-7} M and showed EC_{50} values of 45 ± 6.0 nM (DNA synthesis; $n = 3$) and 60 ± 5.2 nM (nucleus number; $n = 3$). Phenylephrine alone had almost no effect on hepatocyte DNA synthesis and proliferation in the range of 10^{-6} to 10^{-5} M (data not shown). In contrast, the ability of HGF to induce hepatocyte DNA synthesis and proliferation was significantly potentiated by the addition of phenylephrine with the maximal effect seen at a concentration of 10^{-6} M. The potentiation was dose-dependent for phenylephrine up to 10^{-6} M and showed EC_{50} values of 250 ±32 nM (DNA synthesis; $n = 3$) and 600 ± 51 nM (nucleus number; $n = 3$).

Effects of selective *beta*-1 and *beta*-2 adrenergic blockers on metaproterenol-stimulated hepatocyte DNA synthesis and proliferation in the presence of HGF. *Beta* Adrenergic receptors consist of *beta*-1 and *beta*-2 subtypes. Therefore, to further confirm *beta*-2 adrenergic receptor mediation of metaproterenol-stimulated hepatocyte DNA synthesis and proliferation in the presence of 5 ng/ml HGF, we examined the effects of a specific *beta*-1 adrenergic blocker, metoprolol, and a specific *beta*-2 adrenergic blocker, butoxamine, on the potentiation of the HGF effects induced by metaproterenol. As shown in table 2, the effects of metaproterenol were clearly mediated *via* the *beta*-2 adrenergic receptor, because the *beta*-2 selective blocker, butoxamine (10^{-6} M), completely inhibited the metaproterenol effect whereas the *beta*-1 selective blocker, metoprolol (10^{-6} M), had no effect on HGF potentiation during any phase of the primary culture. Metoprolol and butoxamine alone had no direct effects on HGF-stimulated hepatocyte DNA synthesis and proliferation. However, metaproterenol-stimulated hepatocyte DNA synthesis was completely blocked by a nonspecific *beta* adrenergic blocker, propranolol (10^{-6} M), without affecting the HGF response. In addition, stimulation of hepatocyte DNA synthesis and proliferation was not observed with the addition of a *beta*-1 selective agonist, dobutamine (10^{-7}–10^{-5} M), which indicates that potentiation of the HGF effects by metaproterenol is mediated mainly through the *beta*-2 adrenergic receptors.

Effects of H-89 and UK-14304 on metaproterenol- and db-cAMP-stimulated hepatocyte DNA synthesis and proliferation in the presence of HGF. We previously showed that during culture of adult rat hepatocytes, which show a very low *alpha*-2 and *beta* adrenergic response *in vivo*, these responses increase rapidly as a result of the addition of insulin or EGF (Ogihara, 1995, 1996a, b). Based on these findings, we examined the influence of UK14304 (Cambridge, 1981), an *alpha*-2 adrenergic agonist, on metaproterenol- and db-cAMP-stimulated hepatocyte DNA synthesis and proliferation in the presence 5 ng/ml HGF. As shown in table 2, we found that db-cAMP (10^{-7} M) also potentiates hepatocyte DNA synthesis and proliferation induced by HGF. UK14304 (10^{-6} M) inhibited hepatocyte DNA synthesis caused by 10^{-7} M metaproterenol in the presence of 5 ng/ml HGF. In contrast, UK14304 did not affect db-cAMP-stimulated hepatocyte DNA synthesis and proliferation in the presence of HGF. The ability of UK14304 to inhibit metaproterenol-stimulated hepatocyte DNA synthesis was blocked by yohimbine (10^{-5} M; not shown). Each agent alone had no direct effect on either hepatocyte DNA synthesis or proliferation in primary culture.

The isoquinoline sulfonamide, H-89, is known as a specific

TABLE 1

Dose-dependent effects of metaproterenol and phenylephrine on HGF-stimulated hepatocyte DNA synthesis and increase in number of nuclei during early and late phases of culture

Hepatocytes were plated at a density of 3.3×10^4 cells/cm^2. After the attachment period of 3 h, they were cultured for a further 4 h (early phase) and 21 h (late phase) with 5 ng/ml HGF alone or HGF with various concentrations of metaproterenol or phenylephrine. Values are expressed as mean ± S.E. from three independent preparations. Control values for hepatocyte DNA synthesis and the number of nuclei were 568.2 ± 42.6 dpm/mg protein/h and $3.57 \pm 0.15 \times 10^4$ nuclei/cm^2, respectively. Significant differences from HGF alone are indicated by *P < .05.

Treatment	DNA Synthesis		Number of Nuclei	
	Culture time		Culture time	
	4 h	21 h	4 h	21 h
	% of control		*% of control*	
Control	100.0 ± 16.2	103.5 ± 12.2	100.0 ± 4.7	100.2 ± 5.2
HGF, 5 ng/ml	1153.0 ± 129.0	302.6 ± 36.1	133.0 ± 3.3	138.1 ± 3.6
+Metaproterenol (μM)				
0.01	1181.2 ± 142.2	297.9 ± 14.6	133.5 ± 4.5	140.4 ± 4.9
0.02	1196.4 ± 127.0	343.4 ± 24.3	135.3 ± 3.6	142.2 ± 3.0
0.03	1355.9 ± 140.0	518.5 ± 74.8	137.3 ± 3.6	144.3 ± 4.0
0.05	1499.8 ± 142.2	571.4 ± 73.4*	141.4 ± 2.8	147.3 ± 3.7
0.1	1853.6 ± 130.5*	705.9 ± 88.8*	146.8 ± 3.4*	151.0 ± 3.2
0.3	1787.3 ± 155.4*	649.0 ± 78.0*	144.5 ± 4.4	150.0 ± 3.6
1	1546.5 ± 158.3	635.2 ± 69.3*	138.6 ± 5.7	141.7 ± 5.5
+Phenylephrine (μM)				
0.1	1194.4 ± 72.8	308.0 ± 54.3	136.7 ± 6.5	142.4 ± 6.7
0.2	1212.6 ± 98.3	309.5 ± 51.5	138.3 ± 4.6	143.5 ± 3.9
0.3	1697.0 ± 155.4	311.8 ± 62.1	140.7 ± 4.8	145.4 ± 3.5
0.5	1848.7 ± 170.6*	463.6 ± 74.8	147.7 ± 4.0	148.5 ± 3.7
1	2054.6 ± 236.3*	631.7 ± 87.3*	152.3 ± 4.9*	151.2 ± 4.0
3	1884.4 ± 145.7*	620.3 ± 88.8*	148.7 ± 4.2*	150.4 ± 3.2
10	1532.2 ± 147.8	556.4 ± 86.5	143.8 ± 5.9	144.2 ± 4.6

TABLE 2

Effects of specific *beta*-1 and *beta*-2 adrenergic antagonists, UK-14304 and H-89 on metaproterenol- and dibutyryl cAMP-stimulated hepatocyte DNA synthesis and proliferation in the presence of HGF

Hepatocytes were plated at a density of 3.3×10^4 cells/cm^2 and cultured. Specific *beta*-1 and *beta*-2 adrenergic antagonists, UK-14304, H-89, metaproterenol and db-cAMP were added with 5 ng/ml HGF immediately after medium change and cells were cultured for a further 4 h and 21 h. Concentrations were as follows: metaproterenol, 10^{-7} M; propranolol, 10^{-5} M; metoprolol, 10^{-6} M; butoxamine, 10^{-6} M; dobutamine, 10^{-6} M; UK-14304, 10^{-6} M; db-cAMP, 10^{-7} M; H-89, 10^{-7} M. Values are expressed as mean ± S.E. from three independent preparations. Control values for hepatocyte DNA synthesis and the number of nuclei were 573.7 ± 60.2 dpm/mg protein/h and $3.54 \pm 0.15 \times 10^4$ nuclei/cm^2, respectively. Significant differences from HGF alone are indicated by * P < .05.

Treatment	DNA Synthesis		Number of Nuclei	
	Culture time		Culture time	
	4 h	21 h	4 h	21 h
	% of control		*% of control*	
Control	101.0 ± 19.2	100.0 ± 17.8	100.0 ± 4.7	99.9 ± 3.1
HGF	1289.4 ± 119.2	397.1 ± 43.2	132.3 ± 3.2	138.3 ± 3.1
+Metaproterenol	1999.4 ± 164.6*	617.4 ± 51.7*	146.8 ± 4.1*	149.9 ± 3.3
+Propranolol	1283.9 ± 102.9	437.6 ± 42.4	129.8 ± 5.1	139.3 ± 4.8
+Metaproterenol + propranolol	1262.0 ± 147.3	461.2 ± 33.3	131.3 ± 3.4	149.2 ± 5.7
+Metoprolol	1296.8 ± 155.0	352.4 ± 34.1	134.0 ± 4.0	141.3 ± 4.8
+Metaproterenol + metoprolol	1979.4 ± 168.5*	577.1 ± 47.1*	144.1 ± 3.1*	148.5 ± 3.2
+Butoxamine	1309.5 ± 135.7	383.5 ± 43.2	130.6 ± 3.1	139.0 ± 4.4
+Metaproterenol + butoxamine	1219.8 ± 180.7	382.1 ± 22.4	130.6 ± 3.8	139.8 ± 5.0
+Dobutamine	1255.9 ± 123.1	440.8 ± 47.6	130.3 ± 4.4	140.0 ± 3.4
+UK-14304	1305.8 ± 110.9	406.9 ± 44.9	130.4 ± 4.4	139.8 ± 5.2
+Metaproterenol + UK-14304	1225.7 ± 119.7	479.3 ± 34.3	130.8 ± 5.6	140.0 ± 4.9
+db-cAMP	2036.7 ± 167.8*	684.8 ± 59.0*	145.9 ± 4.3*	147.3 ± 4.2
+db-cAMP + UK-14304	1894.8 ± 135.7*	646.6 ± 58.9*	143.8 ± 3.1*	144.0 ± 4.8
+H-89	1279.7 ± 155.9	401.8 ± 43.4	132.9 ± 5.0	137.7 ± 3.9
+Metaproterenol + H-89	1220.1 ± 108.4	432.6 ± 49.6	137.1 ± 4.2	141.3 ± 4.7
+db-cAMP + H-89	1233.4 ± 139.4	382.7 ± 31.3	129.9 ± 5.0	139.9 ± 4.0

inhibitor of the PKA in some cell types (Zusick *et al.*, 1994). Therefore, H-89 is a useful tool to investigate the possible involvement of PKA in the HGF signal transduction pathway. H-89 (10^{-7} M) alone had no significant effect on hepatocyte DNA synthesis and proliferation induced by HGF, which suggests that PKA action *per se* is not sufficient to induce hepatocyte replication. On the other hand, H-89 (10^{-7} M) completely blocked db-cAMP, as well as metaproterenol-stimulated hepatocyte DNA synthesis and proliferation in the presence of HGF (table 2).

Effects of specific *alpha*-1 and *alpha*-2 adrenergic antagonists on the phenylephrine-stimulated hepato-

cyte DNA synthesis and proliferation in the presence of HGF. *Alpha* adrenergic receptors consist of *alpha*-1 and *alpha*-2 subtypes. Therefore, to further confirm *alpha*-1 adrenergic receptor mediation of phenylephrine-stimulated hepatocyte DNA synthesis and proliferation in the presence of 5 ng/ml HGF, we examined the effects of a specific *alpha*-1 adrenergic blocker, prazosin, and a specific *alpha*-2 adrenergic blocker, yohimbine, on the phenylephrine potentiation of the HGF effects. As shown in table 3, the effects of phenylephrine were clearly mediated *via* the *alpha*-1 adrenergic receptors, because the *alpha*-1 selective blocker, prazosin (10^{-6} M), completely inhibited the phenylephrine effect, whereas the *alpha*-2 selective blocker, yohimbine (10^{-6} M), had no effect on phenylephrine action during any phase of the primary culture. Prazosin and yohimbine alone had no direct effects on HGF-stimulated hepatocyte DNA synthesis or proliferation. UK-14304 did not affect phenylephrine-induced hepatocyte DNA synthesis and proliferation, which suggests that *alpha*-2 receptor-mediated mechanism does not couple to PLC.

Effects of U-73122, sphingosine, PMA and ionomycin on the HGF-stimulated hepatocyte DNA synthesis and proliferation in the absence or presence of phenylephrine. We investigated the role of PLC and its intracellular second messengers (*i.e.,* DG and calcium ion) on the HGF-stimulated hepatocyte DNA synthesis and proliferation in the absence or presence of phenylephrine. As shown in table 3, a PLC-γ inhibitor, U-73122 (Thompson *et al.*, 1991), attenuated HGF action on hepatocyte DNA synthesis and proliferation in the absence or presence of phenylephrine. To elucidate whether or not DG, a direct activator of PKC, is involved in the HGF-stimulated hepatocyte DNA synthesis and proliferation, hepatocytes were treated with PMA, a synthetic analog of DG (Castagna *et al.*, 1982), for 4 h and 21 h. PMA (10^{-7} M) alone had no significant effect on hepatocyte DNA synthesis and proliferation, but did potentiate the ability of HGF to stimulate hepatocyte DNA synthesis and proliferation. In addition, U73122 (3×10^{-6} M) attenuated phenylephrine but not PMA action on hepatocyte DNA synthesis and proliferation induced by HGF. Pretreatment of hepatocytes with a PKC inhibitor, sphingosine (10^{-6} M), partially prevented, whereas a higher concentration of sphingosine (3×10^{-6} M) significantly blocked the HGF action on the hepatocyte DNA synthesis and proliferation in the absence or presence of phenylephrine at early and late phases of culture. Each agent alone had no direct effect on either hepatocyte DNA synthesis or proliferation in primary culture. Similarly, to determine the possible involvement of intracellular calcium mobilization in hepatocyte DNA synthesis and proliferation, cells were cultured with 10^{-5} M calcium ionophore (Xiaomei *et al.*, 1995), ionomycin, for 4 h and 21 h. No changes in hepatocyte DNA synthesis and proliferation were observed with the dose of ionomycin. In addition, other calcium-mobilizing agents such as angiotensin II and arginine vasopressin (10^{-8}–10^{-6} M) did not affect hepatocyte DNA synthesis and proliferation induced by HGF (data not shown).

Effect of specific inhibitors of signal-transducing enzymes on hepatocyte DNA synthesis and proliferation induced by HGF with or without agents that elevate

TABLE 3

Effects of specific *alpha*-1 and *alpha*-2 adrenergic antagonists, U-73122, sphingosine and ionomycin on phenylephrine- and phorbol ester-stimulated hepatocyte DNA synthesis and proliferation in the presence of HGF

Hepatocytes were plated at a density of 3.3×10^4 cells/cm^2 and cultured. Specific *alpha*-1 and *alpha*-2 adrenergic antagonists, sphingosine, ionomycin, phenylephrine and PMA were added with 5 ng/ml HGF immediately after medium change, and cells were cultured for a further 4 h and 21 h. Concentrations were as follows: phenylephrine, 10^{-6} M; prazosin, 10^{-6} M; yohimbine, 10^{-6} M; UK-14304, 10^{-6} M; U-73122,[a] 10^{-6} M; U-73122,[b] 3×10^{-6} M; sphingosine,[a] 3×10^{-6} M; sphingosine,[b] 10^{-5} M; ionomycin, 10^{-5} M; PMA, 10^{-7} M. Values are expressed as mean ± S.E. from three independent preparations. Control values for hepatocyte DNA synthesis and the number of nuclei were 546.2 ± 63.2 dpm/mg protein/h and 3.48 ± 0.11 $\times 10^4$ nuclei/cm^2, respectively. Significant differences from HGF alone are indicated by * $P < .05$, ** $P < .01$.

Treatment	DNA Synthesis		Number of Nuclei	
	Culture time		Culture time	
	4 h	21 h	4 h	21 h
	% of control		*% of control*	
Control	102.1 ± 13.1	99.5 ± 10.1	100.7 ± 2.4	99.9 ± 3.7
HGF	1395.1 ± 143.1	375.4 ± 39.1	131.3 ± 3.4	141.6 ± 3.0
+Phenylephrine	2426.0 ± 174.1*	510.2 ± 52.4	143.9 ± 3.0*	149.9 ± 3.0
+Prazosin	1374.1 ± 187.6	334.9 ± 47.2	131.0 ± 3.0	141.3 ± 2.8
+Phenylephrine + prazosin	1298.7 ± 119.4	373.6 ± 39.1	133.1 ± 3.0	138.5 ± 5.2
+Yohimbine	1407.6 ± 122.9	386.2 ± 34.8	130.6 ± 5.1	139.0 ± 4.4
+Phenylephrine + yohimbine	2238.1 ± 184.5*	485.7 ± 38.2	145.6 ± 2.8*	149.8 ± 2.4
+UK-14304	1480.5 ± 107.8	411.1 ± 48.9	130.0 ± 4.1	138.8 ± 4.2
+Phenylephrine + UK-14304	2253.4 ± 159.6*	503.6 ± 40.5	144.1 ± 3.0*	148.5 ± 5.2
+U-73122[a]	951.6 ± 70.7*	608.9 ± 72.0*	119.4 ± 4.0	129.3 ± 3.7
+U-73122[b]	807.4 ± 74.1*	1050.9 ± 99.0**	107.4 ± 3.6**	140.9 ± 4.7
+Phenylephrine + U-73122[b]	1522.0 ± 180.1	401.9 ± 47.7	133.1 ± 3.3	139.3 ± 4.3
+Sphingosine[a]	942.0 ± 73.5*	1111.9 ± 109.5**	120.9 ± 5.4	130.7 ± 3.9
+Sphingosine[b]	703.3 ± 44.6**	1566.2 ± 143.7**	114.6 ± 4.4*	147.7 ± 3.9
+Phenylephrine + sphingosine[b]	1493.5 ± 133.2	388.7 ± 41.4	127.1 ± 4.2	137.3 ± 4.7
+Ionomycin	1635.9 ± 127.0	508.8 ± 40.7	137.3 ± 3.5	144.0 ± 3.4
+Phenylephrine + ionomycin	2326.2 ± 153.8*	463.8 ± 40.4	148.8 ± 3.1*	150.6 ± 3.1
+PMA	2588.8 ± 209.3**	552.6 ± 85.3	152.3 ± 4.2*	153.7 ± 4.5
+PMA + U-73122[b]	2474.7 ± 171.0*	510.8 ± 76.2	149.6 ± 4.0*	151.0 ± 4.6
+PMA + sphingosine[b]	1378.0 ± 221.9	502.6 ± 85.3	130.6 ± 2.0	139.0 ± 4.0
+PMA + ionomycin	2697.3 ± 194.4**	514.7 ± 47.6	154.3 ± 4.4*	154.6 ± 4.0

cAMP. We investigated whether or not the mitogenic responses of hepatocytes to HGF alone and HGF with metaproterenol or phenylephrine are mediated by signal transducers such as receptor tyrosine kinase, PI(3)K or p70 S6K. To determine whether or not HGF-stimulated DNA synthesis and proliferation requires receptor tyrosine kinase activity, hepatocytes were treated with HGF in the presence and absence of a specific tyrosine kinase inhibitor, genistein (Akiyama *et al.*, 1987), for 4 h and 21 h. As shown in table 4, genistein almost completely blocked HGF-induced stimulation of hepatocyte DNA synthesis and the proliferative effects of HGF with or without metaproterenol. Treatment of hepatocytes with a specific PI(3)K inhibitor, wortmannin (10^{-7} M) (Baggiolini *et al.*, 1987; Dewald *et al.*, 1988; Sanchez-Margalet *et al.*, 1994; Ui *et al.*, 1995), also completely inhibited HGF-induced stimulation of hepatocyte DNA synthesis and proliferation in the absence or presence of metaproterenol or phenylephrine. Table 4 also shows that the immunosuppressant, rapamycin (10 ng/ml) (Chung *et al.*, 1992; Price *et al.*, 1992; Downward, 1994), almost completely attenuated both the mitogenic effects of HGF and co-mitogenic effects of metaproterenol and phenylephrine on hepatocyte DNA synthesis and proliferation. The strong mitogenic effects of HGF with metaproterenol or phenylephrine were completely blocked by the addition of a DNA polymerase α inhibitor, aphidicolin (10 μg/ml).

Discussion

We demonstrated that hepatocyte DNA synthesis and proliferation in the primary culture were stimulated 3 to 4 h after the addition of HGF (fig. 1). The mechanisms by which HGF rapidly stimulated hepatocyte DNA synthesis and proliferation may be dependent on dexamethasone in the culture medium, because the rapid stimulatory effects of HGF are dose-dependently inhibited by increasing concentrations of the hormone (fig. 3). The glucocorticoids, dexamethasone and hydrocortisone, have been shown to improve the plating efficiency and viability of hepatocytes, and they are used routinely in primary cultures of adult rat hepatocytes. Some investigators, including the authors, remove the glucocorticoids after an initial attachment period (Ichihara *et al.*, 1980), whereas others maintain the cells in the presence of glucocorticoids during the entire growth stimulatory period (Richman *et al.*, 1976). In addition, they have used relatively large doses of the glucocorticoids (10^{-8}–10^{-6} M) in their cultures. Accordingly, the addition of a low concentration of dexamethasone (*i.e.,* 10^{-10} M) may explain why the results obtained in our short-term studies were different from those of previous extensive studies with longer term culture.

The ability of HGF (5 ng/ml) to induce hepatocyte DNA synthesis and proliferation is strictly dependent on the initial plating density in the presence or absence of metaproterenol (figs. 5 and 6) or phenylephrine (figs. 5 and 6). The mechanisms associated with the density dependence of hepatocyte DNA synthesis and proliferation probably involve cell-to-cell contact (Nakamura *et al.*, 1983a, 1984; Kajiyama and Ui, 1994) and/or the production of inhibitory autocrine factor(s) by hepatocytes in primary culture (Nakamura *et al.*, 1983a). However, further studies are required to confirm this hypothesis.

We have previously demonstrated that cAMP and cAMP-dependent protein kinase (PKA) modulate the regulation of hepatocyte DNA synthesis and proliferation in the presence of EGF (Kimura and Ogihara, 1997a) or insulin (Kimura and Ogihara, in press, 1997b): this conclusion was based on the finding that effects of extracellular application of the cell-permeable cAMP analog, db-cAMP, which directly activates PKA, or the indirect adenylate cyclase activator, metaproterenol, are almost completely blocked by a specific PKA in-

TABLE 4

Effect of specific inhibitors of signal-transducing elements on hepatocyte DNA synthesis and number of nuclei induced by metaproterenol and phenylephrine in the presence of HGF

Hepatocytes were plated at a density of 3.3×10^4 cells/cm^2 and cultured. Specific inhibitors of signal-transducing elements were added with 5 ng/ml HGF immediately after medium change, and cells were cultured for a further 4 h and 21 h. Concentrations were as follows: dexamethasone, 10^{-10} M; metaproterenol, 10^{-7} M; phenylephrine, 10^{-6} M; genistein, 5×10^{-6} M; aphidicolin, 10 μg/ml; wortmannin, 10^{-7} M; rapamycin, 10 ng/ml. Values are expressed as mean ± S.E. from three independent preparations. Control values for hepatocyte DNA synthesis and the number of nuclei were 533.6 ± 59.7 dpm/mg protein/h and 3.62 ± 0.14 $\times 10^4$ nuclei/cm^2, respectively. Significant differences from HGF alone are indicated by * $P < .05$, ** $P < .01$, *** $P < .001$.

Treatment	DNA Synthesis		Number of Nuclei	
	Culture time		Culture time	
	4 h	21 h	4 h	21 h
	% of control		*% of control*	
Control	100.6 ± 13.9	98.7 ± 16.3	100.5 ± 3.0	100.3 ± 4.1
HGF	1319.9 ± 149.5	323.2 ± 45.7	130.4 ± 2.5	140.2 ± 3.0
+Metaproterenol	2042.7 ± 177.6*	703.6 ± 78.5*	145.8 ± 3.2*	146.7 ± 3.1
+Phenylephrine	2531.3 ± 222.4*	510.9 ± 55.1	149.4 ± 3.4*	150.2 ± 4.2
+Genistein	170.0 ± 19.6**	182.0 ± 26.9	103.3 ± 4.1**	103.9 ± 3.6**
+Metaproterenol + genistein	177.2 ± 15.7**	186.8 ± 19.9	104.2 ± 3.5**	108.2 ± 4.4**
+Phenylephrine + genistein	159.8 ± 22.4**	187.8 ± 17.6	106.5 ± 3.6**	104.7 ± 4.7**
+Aphidicolin	12.0 ± 2.6***	10.4 ± 2.9**	100.3 ± 2.1**	100.9 ± 3.0**
+Metaproterenol + aphidicolin	56.8 ± 10.7**	97.3 ± 13.3**	100.6 ± 4.1**	95.2 ± 4.3**
+Phenylephrine + aphidicolin	76.3 ± 9.8**	88.7 ± 10.5**	100.8 ± 3.1**	96.3 ± 3.1**
+Wortmannin	195.0 ± 13.8**	178.1 ± 26.0	102.2 ± 5.5**	100.3 ± 5.0**
+Metaproterenol + wortmannin	223.7 ± 26.0**	279.0 ± 25.9	101.0 ± 5.1**	95.2 ± 4.4**
+Phenylephrine + wortmannin	321.5 ± 29.7**	287.4 ± 21.5	103.8 ± 3.6**	97.4 ± 5.1**
+Rapamycin	109.4 ± 19.4**	156.7 ± 22.0*	100.3 ± 2.1***	110.7 ± 3.7**
+Metaproterenol + rapamycin	139.6 ± 15.3**	176.7 ± 21.9*	104.3 ± 7.1**	106.7 ± 4.4**
+Phenylephrine + rapamycin	149.1 ± 15.8**	160.5 ± 27.3*	100.9 ± 5.8**	103.9 ± 3.2**

hibitor, H-89 (Zuscik *et al.*, 1994). In the present study, hepatocyte proliferation stimulated by metaproterenol or db-cAMP in the presence of HGF was also inhibited by the PKA inhibitor, H-89, suggesting the involvement of PKA (table 2). In addition, the notion of membrane adenylate cyclase involvement is supported by the inhibitory effect of a specific *alpha*-2 adrenergic agonist, UK-14304 (Cambridge, 1981), on metaproterenol-stimulated, but not db-cAMP-stimulated hepatocyte DNA synthesis and proliferation in the presence of HGF. These results can likely be attributed to activation of the adenylate cyclase/PKA pathway. However, the role of the second messenger, cAMP, in the control of hepatocyte DNA synthesis and proliferation remains controversial. Cyclic AMP can either stimulate or inhibit DNA synthesis depending on the culture conditions (Bronstad and Christoffersen, 1980; Bronstad *et al.*, 1983; Mahler and Wilce, 1988; Vintermyr *et al.*, 1989; Refsnes *et al.*, 1992). For example, elevated hepatocyte cAMP levels have been reported to inhibit HGF-stimulated DNA synthesis and proliferation (Marker *et al.*, 1992). In contrast, our results showed that the proliferative effects of HGF are likely, at least in part, to depend on cAMP. Presently, the biological mechanisms by which cAMP modulates hepatocyte DNA synthesis and proliferation remain to be elucidated.

HGF reportedly acts through tyrosine kinase receptors that phosphorylate and activate PLC, which leads to enhanced DG and IP_3 production and mobilization of calcium from intracellular stores (Berridge, 1993; Xiaomei *et al.*, 1995). *Alpha*-1 adrenergic agonists, such as phenylephrine and norepinephrine, exert their action through the activation of PLC-γ. The mechanisms leading to stimulation of hepatocyte DNA synthesis and proliferation by HGF in the absence or presence of phenylephrine have been investigated (table 3) with two mechanistically distinct inhibitors of signal transducers, U73122 (Thompson *et al.*, 1991) and sphingosine (Merrill *et al.*, 1989). The PLC-γ inhibitor, U73122 (3×10^{-6} M), and the PKC inhibitor, sphingosine (3×10^{-6} M), attenuated both mitogenic effects of HGF and co-mitogenic effects of phenylephrine, which suggests that PLC-γ and PKC play an important role in the HGF regulation of hepatocyte DNA synthesis and proliferation. This was supported further by the findings that PMA, a synthetic analog of DG, markedly potentiated the effects of HGF on hepatocyte DNA synthesis and proliferation, and that U73122 attenuated phenylephrine, but not PMA effects on hepatocyte DNA synthesis and proliferation. In agreement with these findings, tyrosine kinase receptors, such as EGF and PDGF, are known to generate IP_3 and DG by interacting directly with PLC-γ to stimulate hepatocyte growth and proliferation (Ullrich and Schlessinger, 1990; Cantley *et al.*, 1991). On the other hand, if the effects of HGF in the absence or presence of phenylephrine were mediated through calcium, the calcium would be replaced by calcium ionophore, ionomycin (Xiaomei *et al.*, 1995). However, calcium ions did not appear to be involved in the HGF effects in the absence or presence of phenylephrine, because no changes in hepatocyte DNA synthesis and proliferation were observed when cells were cultured with 10^{-5} M ionomycin for 4 h and 21 h (table 3). In addition, other calcium-mobilizing agents, such as angiotensin II and arginine vasopressin (10^{-8}–10^{-6} M), did not affect hepatocyte DNA synthesis and proliferation induced by HGF in the absence or presence of phenylephrine (data not shown). Therefore, HGF effects can likely be attributed to activation of the PLC/PKC pathway.

To investigate the possible mechanisms involved in the activation of hepatocyte DNA synthesis and proliferation induced by HGF in primary cultures of adult rat hepatocytes, hepatocytes were cultured with specific inhibitors of signal transducers (table 4). Hepatocyte DNA synthesis and proliferation induced by HGF was almost completely blocked by specific inhibitors of signal transducers, such as a specific tyrosine kinase inhibitor, genistein (Akiyama *et al.*, 1987), a specific PI(3)K inhibitor, wortmannin (Baggiolini *et al.*, 1987; Dewald *et al.*, 1988; Sanchez-Margalet *et al.*, 1994; Ui *et al.*, 1995), and a p70 S6K inhibitor, rapamycin (Chung *et al.*, 1992; Price *et al.*, 1992; Downward, 1994). These results suggest that these signal transducers play an essential role in the mitogenic activity induced by HGF.

In conclusion, the present results demonstrate for the first time that HGF can rapidly induce the proliferation of adult rat hepatocytes in a primary culture. This induction is dependent on the initial plating density. The present results also suggest that after stimulation with HGF, activation of tyrosine kinase, PI(3)K, PLC and P70 S6K is essential for hepatocyte DNA synthesis and proliferation. The mitogenic effects of HGF were potentiated by both a *beta*-2 adrenergic agonist, metaproterenol, which is mediated primarily through PKA and an *alpha*-1 adrenergic agonist, phenylephrine, which is mediated primarily through PKC. Thus, both *alpha*-1 and *beta*-2 adrenergic action may have a positive influence, whereas *alpha*-2 adrenergic action may negatively influence normal liver growth and liver regeneration induced by HGF *in vivo*.

References

Akiyama, T., Ishida, J., Nakagawa, S., Ogawara, H., Watanabe, S., Itoh, N., Shibuya, M. and Fukami, Y.: Genistein, a specific inhibitor of tyrosine-specific protein kinases. J. Biol. Chem. **262:** 5592–5595, 1987.

Baggiolini, M., Dewald, B., Schnyder, J., Ruch, W., Cooper, P. H. and Payne, T. G.: Inhibition of the phagocytosis-induced respiratory burst by the fungal metabolite wortmannin and some analogues. Exp. Cell Res. **169:** 408–418, 1987.

Berridge, M. J.: Inositol trisphosphate and calcium signalling. Nature **361:** 315–325, 1993.

Bronstad, G. and Christoffersen, T.: Increased effect of adrenaline on cyclic AMP formation and positive β-adrenergic modulation of DNA-synthesis in regenerating hepatocytes. FEBS Lett. **120:** 89–93, 1980.

Bronstad, G. O., Sand, T. E. and Christoffersen, T.: Bidirectional concentration-dependent effects of glucagon and dibutyryl cyclic AMP an DNA synthesis in cultured adult rat hepatocytes. Biochim. Biophys. Acta **763:** 58–63, 1983.

Cambridge, D.: UK14304, a potent and selective α2-agonist for the characterisation of α-adrenoceptor subtypes. Eur. J. Pharmacol. **72:** 413–415, 1981.

Cantley, L. C., Auger, K. R., Carpenter, C., Duckworth, B., Graziani, A., Kapeller, R. and Soltoff, S.: Oncogenes and signal transduction. Cell **64:** 281–302, 1991.

Castagna, M., Takai, Y., Kaibuchi, K., Sano, K., Kikkawa, U. and Nishizuka, Y.: Direct activation of calcium-activated, phospholipid-dependent protein kinase by tumor-promoting phorbol esters. J. Biol. Chem. **257:** 7847–7851, 1982.

Chung, J., Kuo, C. D., Crabtree G. R. and Blenis, J.: Rapamycin-FKBP specifically blocks growth-dependent activation of and signaling by the 70 kd S6 protein kinases. Cell **69:** 1227–1236, 1992.

Dewald, B., Thelen, M. and Baggiolini, M.: Two transduction sequences are necessary for neutrophil activation by receptor agonists. J. Biol. Chem. **263:** 16179–16184, 1988.

Downward, J.: Regulating S6 kinase. Nature **371**: 378–379, 1994.

Gines, P., Xiaomei, L., Zamarripa, J. L., Brown, S. E., Wieder, E. D., Nakamura, T., Guzelian, P. S., Schrier, R. W., Heasley, L. E. and Nemenoff, R. A.: Tyrosine kinase growth factor receptors or phorbol esters activate mitogen-activated protein kinase in rat hepatocytes. Hepatology **22:** 1296–1303, 1995.

Ichihara, A., Nakamura, T., Tanaka, K., Tomita, Y., Aoyama, K., Kato, S. and Shinno, H.: Biochemical functions of adult rat hepatocytes in primary culture. Ann. NY Acad. Sci. **349:** 77–84, 1980.

KAJIYAMA, Y. AND UI, M.: Switching from α1- to β-subtypes in adrenergic response during primary culture of adult-rat hepatocytes as affected by the cell-to-cell interaction through plasma membranes. Biochem. J. **303:** 313–321, 1994.

KIMURA, M. AND OGIHARA, M.: Density-dependent proliferation of adult rat hepatocytes in primary culture induced by epidermal growth factor is potentiated by cAMP-elevating agents. Eur. J. Pharmacol. **324:** 267–276, 1997a.

KIMURA, M. AND OGIHARA, M.: Proliferation of adult rat hepatocytes in primary culture induced by insulin is potentiated by cAMP-elevating agents. Eur. J. Pharmacol., in press, 1997b.

LEE, M. B. AND PAXMAN, S.: Modification of the Lowry procedure for the analysis of proteolipid protein. Anal. Biochem. **47:** 184–192, 1972.

MAHLER, S. M. AND WILCE, P. A.: Desensitization of adenylate cyclase and cyclic AMP flux during the early stages of liver regeneration. J. Cell. Physiol. **136:** 88–94, 1988.

MARKER, A., GALLOWAY, J., E., PALMER, S., NAKAMURA, T., GOULD, G. W., MACSWEEN R. N. M. AND BUSHFIELD, M.: Role of the adenylate cyclase, phosphoinositidase C and receptor tyrosyl kinase systems in the control of hepatocyte proliferation by hepatocyte growth factor. Biochem. Pharmacol. **44:** 1037–1043, 1992.

MCGOWAN, J. A., STRAIN, A. J. AND BUCHER, N. R.: DNA synthesis in primary cultures of adult rat hepatocytes in a defined medium: Effects of epidermal growth factor, insulin, glucagon, and cyclic-AMP. J. Cell. Physiol. **108:** 353–363, 1981.

MERRILL, JR. A. H., NIMKAR, S., MENALDINO, D., HANNUN, Y. A., LOOMIS, C., BELL, R. M., TYAGI, S. R., LAMBETH, J. D., STEVENS, V. L., HUNTER, R. AND LIOTTA, D. C.: Structural requirements for long-chain (sphingoid) base inhibition of protein kinase C *in vitro* and for the cellular effects of these compounds. Biochemistry **28:** 3138–3145, 1989.

MICHALOPOULOS, G. K.: Liver regeneration; molecular mechanisms of growth control. FASEB J. **4:** 176–187, 1990.

MORLEY, C. G. D. AND KINGDON, H. S.: Use of ^{3}H-thymidine for measurement of DNA synthesis in rat liver: a warning. Anal. Biochem. **45:** 298–305, 1972.

NAKAMURA, T., TERAMOTO, H. AND ICHIHARA, A.: Purification and characterization of a growth factor from rat platelets for mature parenchymal hepatocytes in primary cultures. Proc. Natl. Acad. Sci. U.S.A. **83:** 6489–6493, 1986.

NAKAMURA, T., NAWA, K., ICHIHARA, A., KAISE, N. AND NISHINO, Y.: Purification and subunit structure of hepatocyte growth factor from rat platelets. FEBS Lett. **224:** 311–316, 1987.

NAKAMURA, T., TOMITA, Y. AND ICHIHARA, A.: Density-dependent growth control of adult rat hepatocytes in primary culture. J. Biochem. **94:** 1029–1035, 1983a.

NAKAMURA, T., NISHIZAWA, T., HAGIYA, M., SEKI, T., SHIMONISHI, M., SUGIYAMA, A., TASHIRO, K. AND SHIMIZU, S.: Molecular cloning and expression of human hepatocyte growth factor. Nature **342:** 440–443, 1989.

NAKAMURA, T., YOSHIMOTO, K., NAKAYAMA, Y., TOMITA, Y. AND ICHIHARA, A.: Reciprocal modulation of growth and differentiated functions of mature rat hepatocytes in primary culture by cell-cell contact and cell membranes. Proc. Natl. Acad. Sci. U.S.A. **80:** 7229–7233, 1983b.

NAKAMURA, T., NAKAYAMA, Y. AND ICHIHARA, A.: Reciprocal modulation of growth and liver functions of mature rat hepatocytes in primary culture by an extract of hepatic plasma membranes. J. Biol. Chem. **259:** 8056–8058, 1984.

OGIHARA, M.: Expression of α2-receptor-mediated responses by insulin in primary culture of rat hepatocytes. Jpn. J. Pharmacol. **68:** 11–18, 1995.

OGIHARA, M.: Cell-density-dependent expression of the α2-adrenergic response by epidermal growth factor (EGF) in primary cultures of adult rat hepatocytes. Biol. Pharm. Bull. **19:** 518–524, 1996a.

OGIHARA, M.: Cell-density-dependent expression of the β-adrenergic response by epidermal growth factor (EGF) in primary cultures of adult rat hepatocyte. Biol. Pharm. Bull. **19:** 752–757, 1996b.

OSADA, S., NAKASHIMA, S., SAJI, S., NAKAMURA, T., NOZAWA, Y.: Hepatocyte growth factor (HGF) mediates the sustained formation of 1, 2-diacylglycerol via phosphatidylcholine-phospholipase in cultured rat hepatocytes. FEBS Lett. **297:** 271–274, 1992.

PRICE, D. J., GROVE, J. R., CALVO, V., AVRUCH, J. AND BIERER, B. E.: Rapamycin-induced inhibition of the 70-kilodalton S6 protein kinase. Science **257:** 973–977, 1992.

REFSNES, M., THORESEN, G. H., SANDNES, D., DAJANI, O. F., DAJANI, L. AND CHRISTOFFERSEN, T.: Stimulatory and inhibitory effects of catecholamines on DNA synthesis in primary rat hepatocyte cultures: Role of Alpha$_1$-and beta-adrenergic mechanisms. J. Cell. Physiol. **151:** 164–171, 1992.

RICHMAN, R. A., CLAUS, T. H., PILKIS, S. J. AND FRIEDMANN, D. L.: Hormonal stimulation of DNA synthesis in primary cultures of adult rat hepatocytes. Proc. Natl. Acad. Sci. U.S.A. **73:** 3589–3593, 1976.

SANCHEZ-MARGALET, V., GLDOFINE, I. D., VLAHOS, C. J. AND SUNG, C. K.: Role of phosphatidylinositol-3-kinase in insulin receptor signaling: Studies with inhibitor, LY 294002. Biochem. Biophys. Res. Commun. **204:** 446–452, 1994.

SANDNES, D., SAND, T. E., SANGER, G., BRONSTAD, G. O., REFSNES, M. R., GLADHAUG, I. P., JACOBSEN, S. AND CHRISTOFFERSEN, T.: Elevated level of β-adrenergic receptors in hepatocytes from regenerating rat liver. Exp. Cell Res. **165:** 117–126, 1986.

SEGLEN, P. O.: Preparation of isolated liver cells. Meth. Cell Biol. **13:** 29–83, 1975.

THOMPSON, A. K., MOSTAFAPOUR, S. P., DENLINGER, L. C., BLEASDALE, T. E. AND FISHER, S. K.: The aminosteroid U-73122 inhibits muscarinic receptor sequestration and phosphoinositide hydrolysis in SK-N-SH neuroblastoma cells. J. Biol. Chem. **35:** 23856–23862, 1991.

UI, M., OKADA, T., HAZEKI, K. AND HAZEKI, O.: Wortmannin as a unique probe for an intracellular signalling protein, phosphoinositide 3-kinase. Trends Biochem. Sci. **20:** 303–307, 1995.

ULLRICH, A. AND SCHLESSINGER, J.: Signal transduction by receptors with tyrosine kinase activity. Cell **61:** 203–212, 1990.

VINTERMYR, O. K., MELLGREN, G., BOE, R. AND DOSKELAND, T. O.: Cyclic adenosine monophosphate acts synergistically with dexamethasone to inhibit the entrance of cultured adult rat hepatocyte into s-phase with a note on the use of nucleolar and extranucleolar [^{3}H]thymidine labelling patterns to determine rapid changes in the rate of onset of DNA replication. J. Cell Physiol. **141:** 371–382, 1989.

XIAOMEI LI, P. G., ZAMARRIPA, J. L., BROWN, S. E. S., WIEDER, E. D., NAKAMURA, T., GUZELIAN, P. S. SCHRIER, R. W., HEASLEY, L. E. AND NEMENOFF, R. A.: Tyrosine kinase growth factor receptors but not seven-membrane-spanning receptors or phorbol esters activate mitogen-activated protein kinase in rat hepatocytes. Hepatology **22:** 1296–1303, 1995.

ZUSCIK, M. J., PUZAS, J. E., ROSIER, R. N., GUNTER, K. K. AND GUNTER, T. E.: Cyclic-AMP-dependent protein kinase activity is not required by parathyroid hormone to stimulate phosphoinositide signaling in chondrocytes but is required to transduce the hormone's proliferative effect. Arch. Biochem. Biophys. **315:** 352–361, 1994.

Send reprint requests to: Masahiko Ogihara, Ph. D., Biochemical Pharmacology Group, Faculty of Pharmaceutical Sciences, Josai University. 1–1, Keyakidai, Sakado, Saitama 350–02 Japan.