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Effects of insulin-like growth factor I and II on DNA synthesis and proliferation in primary cultures of adult rat hepatocytes

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Abstract

We compared the effects of insulin-like growth factor I (IGF-I) and II (IGF-II) on DNA synthesis and proliferation and investigated various signal transduction mechanisms involved in insulin-like growth factor-induced mitogenesis in primary cultures of adult rat hepatocytes. IGF-I stimulated hepatocyte DNA synthesis and proliferation with an EC_{50} of 75 ng/ml within 4 h of culture. These effects were sensitive to the IGF-I concentration and cell density. Hepatocyte proliferation induced by IGF-I was potentiated by metaproterenol (10^{-6} M) as well as by 8-bromo-cAMP, phorbol 12-myristate 13-acetate (PMA; 10^{-8} M) and was inhibited by U-73122 (1-(-[[17β-3-methoxyestra-1, 3, 5 (10)-triene-17-yl] amino] hexyl]-1*H* pyrrol-2, 5-dione)), genistein, wortmannin, PD98059 (2′-amino-3′-methoxyflavone) and rapamycin. The IGF-I effect was independent of pertussis toxin (100 ng/ml). IGF-II also dose dependently stimulated hepatocyte DNA synthesis and proliferation with an EC_{50} of 0.75 ng/ml within 4 h of culture. However, these effects were not dependent on the initial plating density. The stimulatory effects of IGF-II were potentiated by UK-14304 (5-bromo-6-[2-imidazolin-2-ylamino]-quinoxaline) (10^{-5} M) and inhibited by phenylephrine, PMA, metaproterenol, 8-bromo-cAMP, PD98059, rapamycin, and pertussis toxin. The IGF-II effects were not affected by genistein, U-73122, and wortmannin. These results suggest that IGF-I and IGF-II rapidly stimulate the DNA synthesis and proliferation of adult rat hepatocytes by separate mechanisms.

Keywords: IGF-I (insulin-like growth factor I); IGF-II (insulin-like growth factor II); Adrenoceptor agonist; DNA synthesis; Hepatocyte proliferation

1. Introduction

Hepatocyte proliferation in vivo is generally controlled by several growth factors with stimulatory and inhibitory activities (Michalopoulos, 1990). The key to defining these processes may lie in understanding the actions of these growth factors and the interactions among them. Such activities in vitro can be studied in primary cultures. For example, we recently demonstrated that epidermal growth factor (EGF) (Kimura and Ogihara, 1997a), insulin (Kimura and Ogihara, 1997b), hepatocyte growth factor (HGF) (Kimura and Ogihara, 1997c) and platelet-derived growth factor (PDGF) (Kimura and Ogihara, 1998) can rapidly stimulate hepatocyte DNA synthesis and proliferation during short-term culture (about 3–4 h). The rapid proliferative responses of hepatocytes to these growth factors were dependent on the culture conditions (for example, hormones in the culture medium and plating density). Therefore, we investigated whether or not other growth factors, such as insulin-like growth factors (IGFs), also stimulate DNA synthesis and proliferation in primary cultures of adult rat hepatocytes.

The insulin-like growth factors are a family of polypeptide hormones that have close structural and functional homology with insulin (Humbel, 1990). The IGF peptides act as autocrine growth factors and are implicated in the regulation of fetal growth and in the development of hepatocytes and other cell lines in the culture (Lund et al., 1986; Alphons et al., 1995). The physiological actions of type I IGF (IGF-I) and type II IGF (IGF-II) are mediated by specific interactions with cell surface receptors. Their physiological roles are determined by the distribution of receptors for them on the cell surface and/or the pharmacodynamics of the two factors (Beguinot et al., 1985; Lee et al., 1986; Burant et al., 1987; Steele-Perkins et al., 1988;

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Rosenthal et al., 1991; Christofori et al., 1994). However, the role of IGF peptides in the proliferation of adult rat hepatocytes is poorly understood and the signal transduction mechanisms involved in this event are not well defined (Morgan et al., 1987; Czech, 1989; Melmed et al., 1996). We therefore compared the effects of IGF-I and IGF-II on hepatocyte DNA synthesis and proliferation, and the modulation of these effects by α- and β-adrenoceptor agonists in primary culture. To clarify how IGFs induce hepatocyte DNA synthesis and proliferation, we further examined the effects of specific inhibitors of signal transducers on these responses in primary cultures of adult rat hepatocytes.

Our results show that IGF-I and IGF-II can stimulate hepatocyte DNA synthesis and proliferation during short-term culture (about 3–4 h). IGF-II more potently promoted hepatocyte DNA synthesis and proliferation than IGF-I did. The effects of IGF-I and IGF-II were modulated by different α- and β-adrenoceptor agonists. In addition, studies using specific inhibitors of signal transducing elements demonstrated that the signal transducing pathways of IGF-I and IGF-II are distinct.

2. Materials and methods

2.1. Hepatocyte isolation and culture

Male Wistar rats (200–250 g) obtained from Saitama Experimental (Saitama, Japan) were anesthetized by an intraperitoneal injection of sodium pentobarbital (45 mg/kg). Disaggregation of the liver was facilitated by two-step in-situ collagenase perfusion as described (Seglen, 1975). The viability of hepatocytes consistently exceeded 97% as determined by Trypan blue exclusion. Unless otherwise indicated, isolated hepatocytes were plated onto collagen-coated plastic culture dishes (35 mm diameter; Sumitomo Bakelite, Tokyo, Japan) at a density of 3.3×10^4 cells/cm^2 in Williams' medium E containing 5% bovine calf serum and 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 IGF-I and IGF-II with or without cAMP-elevating agents, PMA, an α_1-adrenoceptor agonist, an α_2-adrenoceptor agonist, and/or specific inhibitors of signal transducers.

2.2. Measurement of DNA synthesis

Hepatocyte DNA synthesis was assessed by measuring the incorporation of [^{3}H]thymidine into acid-precipitable materials. Briefly, after an initial attachment period of 3 h, hepatocytes were washed twice with serum-free Williams' medium E and cultured in medium containing 100 ng/ml IGF-I and -II (1.0 ng/ml) for a further 4 and 21 h. The cells were pulsed at 2 and 19 h post-IGF-I and IGF-II stimulation for 2 h with [^{3}H]thymidine (1.0 μCi/well). Incorporation of [^{3}H]thymidine into DNA was then determined as described (Morley and Kingdon, 1972). Hepatocyte protein content was measured by a modified Lowry procedure using bovine serum albumin as a standard (Lee and Paxman, 1972).

2.3. Counting nuclei

Nuclei instead of cells were counted according to Nakamura et al. with minor modifications (Nakamura et al., 1983a,b). Briefly, primary cultured hepatocytes were washed twice with 2 ml of Dulbecco's phosphate-buffered saline (pH 7.4), and then 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 (pH 7.4) and nuclei were counted in a hemocytometer. This procedure was performed because the hepatocytes firmly attached to the collagen-coated plates and were not sufficiently dispersed by 0.02% EDTA–0.05% trypsin (Kimura and Ogihara, 1997a).

2.4. Materials

The following reagents were obtained from Sigma (St. Louis, MO, USA): 8-bromo cAMP (8-br-cAMP), genistein, aphidicolin, metaproterenol hemisulfate, phenylephrine hydrochloride, glucagon, forskolin, dobutamine hydrochloride, D-sphingosine, dexamethasone and pertussis toxin. Recombinant human insulin-like growth factor I, II, wortmannin, rapamycin, and PD98059 (2′-amino-3′-methoxyflavone) were obtained from R&D Systems (Minneapolis, MN, USA). Phorbol 12-myristate 13-acetate (PMA) was purchased from Research Biochemicals International (Natick, MA, USA). U-73122 (1-(-[[17β-3-methoxyestra-1, 3, 5 (10)-triene-17-yl] amino] hexyl]-1*H* pyrrol-2, 5-dione)), U-73343 (1- [6-[[17β-3-methoxyestra-1, 3, 5 (10)-triene-17-yl] amino] hexyl]-2, 5-pyrrolidinedione), and H-89 · 2HCl (*N*-[2-(*p*-bromocinnamylamino) ethyl]-5-isoquinolinesulfonamide dihydrochloride) were obtained from BIOMOL Research Laboratories, (Plymouth Meeting, PA, USA). UK-14304 (5-bromo-6-[2-imidazolin-2-ylamino]-quinoxaline) was a gift from Pfizer Central Research (Sandwich, UK). Williams' medium E and newborn calf serum were purchased from Flow Laboratories (Irvine, UK). Collagenase (type II) was obtained from Worthington Biochemical, (Freehold, NJ, USA). [Methyl-^{3}H]thymidine (20 Ci/mmol) was obtained from DuPont-New England Nuclear (Boston, MA, USA). All other reagents were of analytical grade.

2.5. Statistical analysis

Data are expressed as means ± S.E.M. Data were analyzed by the unpaired Student's *t*-test. *P*-values below 0.05 were regarded as statistically significant.

3. Results

3.1. Time course of the stimulation of hepatocyte DNA synthesis and proliferation induced by IGF-I and IGF-II

We compared the actions of IGF-I and IGF-II on hepatocyte DNA synthesis and the number of nuclei (proliferation). Fig. 1 shows that exogenous IGF-I (100 ng/ml) or IGF-II (1.0 ng/ml) promoted hepatocyte DNA synthesis and proliferation. Hepatocyte DNA synthesis significantly increased 2.5 and 1.5 h after culture with IGF-I and IGF-II, respectively. Hepatocyte DNA synthesis was markedly reduced after exposure to IGF-I for 21 h. The number of nuclei (proliferation) induced by IGF-I (100 ng/ml) significantly increased about 3.5 h after IGF-I addition, reached a peak at 4 h and remained at this level for a further 17 h. Hepatocyte proliferation induced by IGF-II (1.0 ng/ml) significantly increased about 3.0 h after IGF-II addition, reached a plateau at 4 h and remained at this level for a further 17 h. In general, the ability of IGFs to stimulate hepatocyte DNA synthesis correlated with the number of nuclei (proliferation).

3.2. Dose-dependent effects of IGF-I and IGF-II on hepatocyte DNA synthesis and proliferation

We examined the dose-dependent effects of IGF-I and IGF-II on hepatocyte DNA synthesis and proliferation. Hepatocytes were incubated with various concentrations of IGF-I and IGF-II for 4 h, and then DNA synthesis and proliferation were measured. Fig. 2 shows that both IGF-I and IGF-II dose dependently stimulated the rate of hepatocyte DNA synthesis and proliferation. DNA synthesis was maximally increased by 100 ng/ml IGF-I and by 1.0 ng/ml IGF-II. The 50% effective concentration (EC_{50}) was 75 and 0.75 ng/ml of IGF-I and IGF-II, respectively. Hepatocyte proliferation was maximally increased by 100 and 20 ng/ml of IGF-I and IGF-II, respectively. Therefore, IGF-II more potently stimulated hepatocyte DNA synthesis and proliferation than IGF-I did.

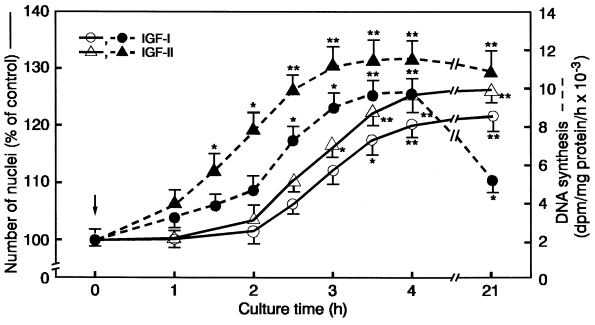

Fig. 1. Time-course associated with stimulation of hepatocyte DNA synthesis and proliferation induced by IGF-I and IGF-II. Hepatocytes at a density of 3.3×10^4 cells/cm^2 were plated and cultured in Williams' medium E containing 5% newborn calf serum and 0.1 nM dexamethasone for 3 h. After an attachment period of 3 h (zero time), the medium was rapidly replaced with serum- and dexamethasone-free Williams' medium E and the hepatocytes were cultured with 100 ng/ml IGF-I or 1.0 ng/ml IGF-II for additional time periods. Hepatocyte DNA synthesis (dotted lines) and proliferation (solid lines) were determined as described in Section 2. Data are expressed as means ± S.E.M. of three separate experiments. $*P<0.05$, $**P<0.01$ compared with respective control.

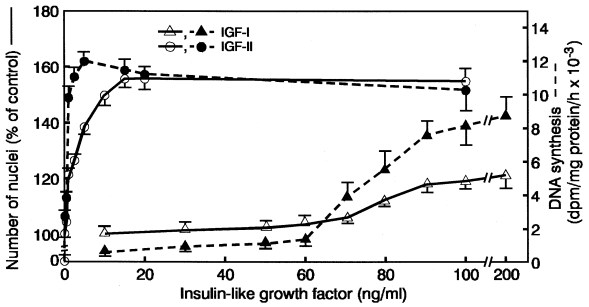

Fig. 2. Dose-dependent effects of IGF-I and IGF-II on hepatocyte DNA synthesis and proliferation. Hepatocytes at a density of 3.3×10^4 cells/cm^2 were plated and cultured for 3 h as described in the legend to Fig. 1. After an attachment period of 3 h (zero time), the medium was rapidly replaced with serum- and dexamethasone-free Williams' medium E and the hepatocytes were cultured with various concentrations of IGF-I or IGF-II for additional time periods. Hepatocyte DNA synthesis (dotted lines) and proliferation (solid lines) were determined as described in Section 2. Data are expressed as means ± S.E.M. of three separate experiments.

As to the receptor specificity for each growth factor, we did not directly examine IGF-I and IGF-II receptor levels (and binding) in primary cultured hepatocytes. However, we actually found that the proliferative effects of IGF-I (100 ng/ml) on hepatocyte DNA synthesis and proliferation were completely blocked by a monoclonal antibody against IGF-I receptor (1:2000–1000 dilution; Calbiochem, USA), whereas the effects of IGF-II (1 ng/ml) were not affected. Conversely, the proliferative effects of IGF-II (1 ng/ml) on hepatocyte DNA synthesis and proliferation were almost completely abolished by a monoclonal antibody against the IGF-II receptor (1:2000–1000 dilution, Austral Biochemicals, USA), whereas the effects of IGF-I (100 ng/ml) were not significantly blocked (Kimura and Ogihara, unpublished observation). These results suggest that the proliferative effect of each growth factor is largely mediated by binding to its own receptor.

3.3. Influence of cell density on hepatocyte DNA synthesis and proliferation stimulated by IGF-I and IGF-II

To determine whether or not the proliferative effects of IGF-I and IGF-II are affected by the initial plating density, we investigated the density-dependence of hepatocyte DNA

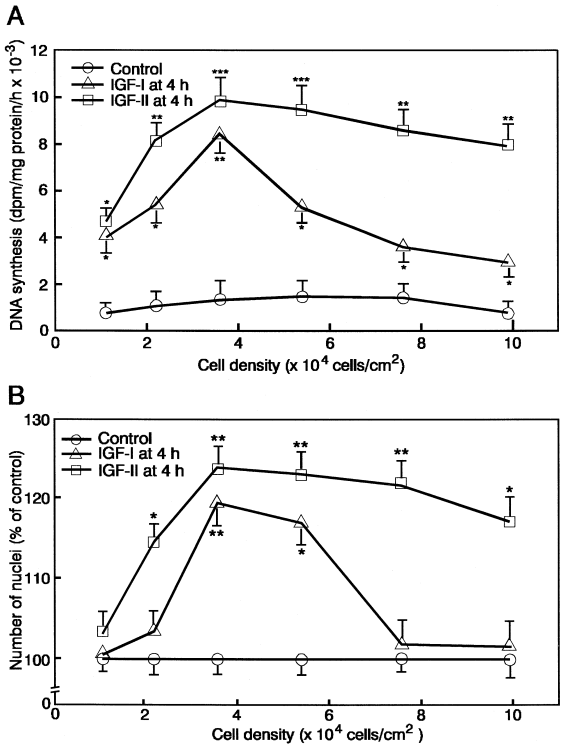

Fig. 3. Influence of cell density on IGF-I- and IGF-II-stimulated hepatocyte DNA synthesis and proliferation. Hepatocytes at various cell densities were cultured with 100 ng/ml IGF-I or 1.0 ng/ml IGF-II for 4 h after the medium was changed (see legend to Fig. 1). Hepatocyte DNA synthesis (dotted lines) and proliferation (solid lines) were determined as described in Section 2. Data are expressed as means ± S.E.M. of three separate experiments. $*P < 0.05$, $**P < 0.01$ compared with respective control.

synthesis and proliferation induced by the IGFs at 4 h of culture. Fig. 3A shows that hepatocyte DNA synthesis induced by IGF-I (100 ng/ml) decreased markedly as the initial plating density increased. In contrast, IGF-II-induced hepatocyte DNA synthesis was not significantly affected by the initial plating density. Hepatocyte proliferation induced by IGF-I (100 ng/ml) reached a maximum at cell densities of 3.3×10^4 and 5.0×10^4 cells/cm^2 (Fig. 3B). Proliferation was almost completely inhibited at initial cell densities of 7.0×10^4 and 1.0×10^5 cells/cm^2. Fig. 3B showed that the number of nuclei induced by 1.0 ng/ml IGF-II reached a plateau at a cell density of 3.3×10^4 cells/cm^2 and was not essentially affected by the initial plating density.

3.4. Effect of metaproterenol and UK-14304 on IGF-I and IGF-II-induced hepatocyte DNA synthesis and proliferation

We investigated factors that functionally interact with IGF signal transduction pathways. We examined the ability of the β_2-adrenoceptor agonist, metaproterenol, to affect IGF-I or IGF-II-induced hepatocyte DNA synthesis and proliferation at 4 h of culture. Fig. 4A shows that the hepatocyte DNA synthesis and proliferation induced by 100 ng/ml IGF-I were dose dependently potentiated by metaproterenol with an EC_{50} value of 5×10^{-7} M. In contrast, 1.0 ng/ml IGF-II-induced hepatocyte DNA synthesis and proliferation were almost completely inhibited by metaproterenol with an IC_{50} value of 7×10^{-8} M. The effects of metaproterenol on IGF-induced hepatocyte DNA synthesis closely correlated with the level of hepatocyte proliferation, as assessed by the number of nuclei.

We then examined the ability of the specific α_2-adrenoceptor agonist, UK-14304 (Cambridge, 1981; Ogihara, 1995), to affect the IGF-I or IGF-II-induced hepatocyte DNA synthesis and proliferation after 4 h of culture. Fig. 4B shows that hepatocyte DNA synthesis induced by 100 ng/ml of IGF-I was not affected by UK-14304. In contrast, the hepatocyte DNA synthesis induced by 1.0 ng/ml

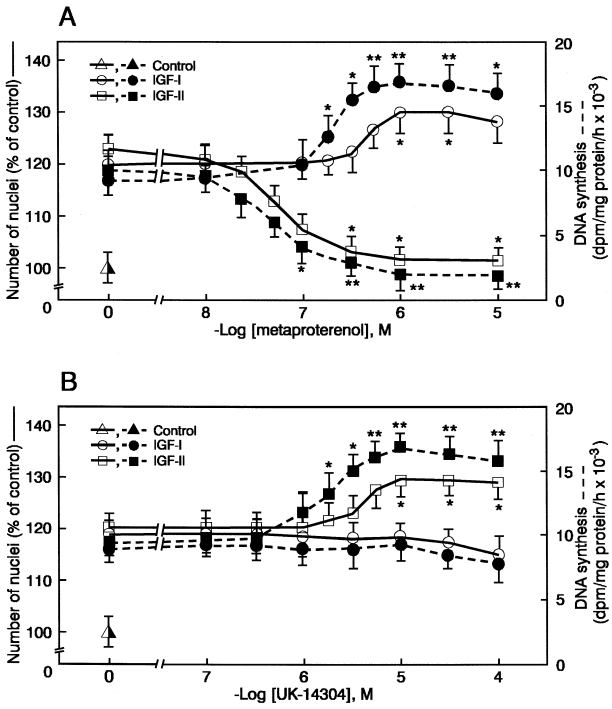

Fig. 4. Effects of metaproterenol and UK-14304 on IGF-I and IGF-II-induced hepatocyte DNA synthesis and proliferation. Hepatocytes at a density of 3.3×10^4 cells/cm^2 were plated and cultured for 3 h as described in the legend to Fig. 1. After an attachment period of 3 h (zero time), the medium was rapidly replaced with serum- and dexamethasone-free Williams' medium E and the hepatocytes were cultured with various concentrations of metaproterenol and/or UK-14304 in the presence of 100 ng/ml IGF-I or 1.0 ng/ml IGF-II for an additional 4 h. Hepatocyte DNA synthesis (dotted lines) and proliferation (solid lines) were determined as described in Section 2. Data are expressed as means ± S.E.M. of three separate experiments. $*P < 0.05$, $**P < 0.01$ compared with respective control.

IGF-II was dose dependently potentiated by UK-14304 with an EC_{50} value of 5×10^{-6} M. The effects of UK-14304 on IGF-I and -II-induced hepatocyte DNA synthesis closely correlated with the level of hepatocyte proliferation. The potentiating effects of UK-14304 were blocked by the specific α_2-adrenoceptor antagonist, yohimbine (10^{-7} M), suggesting mediation by the α_2-adrenoceptor (data not shown).

3.5. Effects of H-89 on hepatocyte DNA synthesis and proliferation induced by cAMP-elevating agents in the presence of IGF-I or IGF-II

To further characterize the involvement of the cAMP/protein kinase A system in IGF-I- or IGF-II-induced hepatocyte DNA synthesis and proliferation, we investigated the effects of the specific protein kinase A inhibitor, H-89 (Zusick et al., 1994), on these responses. Table 1 shows that 10^{-7} M glucagon and 10^{-7} M forskolin, which increase adenylate cyclase activity by different mechanisms, potentiated the DNA synthesis and proliferation induced by 100 ng/ml IGF-I in hepatocytes cultured at 4 and 21 h. These cAMP-elevating agents alone did not affect the responses (data not shown). Consistent with these results, the cell-permeable cAMP analog, 8-bromo cAMP (8-br-cAMP; 10^{-7} M), also potentiated IGF-I-induced hepatocyte DNA synthesis and proliferation at 4 h of culture. The specific protein kinase A inhibitor, H-89 (10^{-7} M), blocked the stimulatory effects of 10^{-6} M metaproterenol and 10^{-7} M 8-br-cAMP on hepatocyte DNA synthesis and proliferation in the presence of 100 ng/ml IGF-I. The stimulation of IGF-I-induced hepatocyte DNA synthesis and proliferation produced by metaproterenol was abolished by UK-14304, suggesting that adenylate cyclase activity is inhibited via G_i-proteins in cultured hepatocytes. In contrast, 10^{-7} M metaproterenol and other cAMP-elevating agents (10^{-7} M glucagon, 10^{-7} M forskolin, and 10^{-7} M 8-br-cAMP) almost completely

Table 1
Effects of H-89 in the presence of IGF-I or IGF-II on hepatocyte DNA synthesis and proliferation induced by cAMP-elevating agents

Agent	DNA synthesis (dpm/mg protein per h $\times 10^{-3}$)		Number of nuclei (% of control)	
	Culture time (h)		Culture time (h)	
	4	21	4	21
Control	1.965 ± 0.210	1.090 ± 0.338	100.6 ± 3.4	99.8 ± 3.7
IGF-I	8.706 ± 1.013[b]	5.417 ± 1.213[a]	121.1 ± 1.6[b]	122.7 ± 1.4[b]
+ UK-14304	8.574 ± 2.323[a]	4.945 ± 1.079[a]	120.9 ± 2.1[b]	121.0 ± 3.5[a]
+ H-89	8.655 ± 1.206[b]	5.402 ± 1.290[a]	120.9 ± 1.4[b]	120.7 ± 2.9[a]
+ Metaproterenol	14.560 ± 1.382[b,c]	13.044 ± 1.083[b,d]	130.8 ± 3.1[b,c]	131.4 ± 2.2[b,c]
+ Metaproterenol + UK-14304	8.834 ± 1.016[b]	6.067 ± 1.018[b]	120.3 ± 5.1[a]	120.0 ± 3.9[a]
+ Metaproterenol + H-89	7.894 ± 1.671[a]	5.586 ± 1.908	119.1 ± 2.2[a]	120.3 ± 2.7[b]
+ 8-Bromo cAMP	18.187 ± 1.318[b,d]	13.807 ± 1.044[b,d]	132.4 ± 2.3[b,c]	133.5 ± 2.8[b,c]
+ 8-Bromo cAMP + UK-14304	16.887 ± 2.018[b,c]	13.905 ± 1.302[b,d]	133.2 ± 2.7[b,c]	134.6 ± 2.0[b,d]
+ 8-Bromo cAMP + H-89	7.673 ± 1.731[a]	5.287 ± 1.372	119.6 ± 2.6[a]	119.9 ± 2.0[b]
+ Glucagon	12.927 ± 1.003[b,c]	11.030 ± 1.020[b,c]	126.9 ± 1.3[b,c]	127.9 ± 1.3[b]
+ Forskolin	16.180 ± 1.080[b,d]	12.239 ± 1.174[b,c]	130.1 ± 2.6[b,c]	130.6 ± 2.4[b,c]
+ Dobutamine	8.588 ± 1.396[a]	5.350 ± 1.054[a]	120.3 ± 2.4[b]	120.0 ± 3.2[a]
IGF-II	11.277 ± 1.013[b]	10.090 ± 1.020[b]	122.6 ± 3.5[a]	123.2 ± 3.2[b]
+ UK-14304	18.572 ± 1.320[b,e]	17.745 ± 1.079[b,f]	133.9 ± 2.0[b,e]	134.0 ± 2.1[b,e]
+ H-89	9.754 ± 1.206[b]	9.408 ± 1.198[b]	120.9 ± 2.2[b]	121.1 ± 3.7[a]
+ Metaproterenol	3.530 ± 0.682[f]	3.044 ± 0.883[f]	104.8 ± 3.1[e]	105.2 ± 2.9[e]
+ Metaproterenol + UK-14304	9.034 ± 0.916[b]	9.867 ± 1.018[b]	123.3 ± 3.1[a]	124.0 ± 2.9[b]
+ Metaproterenol + H-89	9.833 ± 1.476[b]	9.585 ± 1.643[b]	119.1 ± 2.2[b]	121.3 ± 2.3[b]
+ 8-Bromo cAMP	2.187 ± 0.618[f]	2.107 ± 1.344[e]	102.4 ± 4.4[e]	101.5 ± 5.8[e]
+ 8-Bromo cAMP + UK-14304	2.487 ± 1.018[f]	2.076 ± 1.306[f]	103.2 ± 3.0[e]	103.6 ± 3.0[e]
+ 8-Bromo cAMP + H-89	10.673 ± 1.832[a]	9.287 ± 1.577[b]	118.6 ± 4.8[a]	119.9 ± 2.3[b]
+ Glucagon	2.927 ± 1.103[f]	2.403 ± 1.120[f]	103.9 ± 3.0[e]	104.9 ± 5.3[e]
+ Forskolin	3.469 ± 1.089[f]	2.839 ± 1.014[f]	104.1 ± 3.2[e]	104.6 ± 3.4[e]
+ Dobutamine	9.588 ± 1.593[a]	9.350 ± 1.254[b]	120.3 ± 3.4[a]	120.6 ± 3.2[a]

Hepatocytes were plated at a density of 3.3×10^4 cells/cm^2 and cultured for 4 and 21 h with 100 ng/ml IGF-I alone or 1 ng/ml IGF-II alone or with various cAMP-elevating agents immediately after the medium was changed. UK-14304, 10^{-5} M; H-89, 10^{-7} M; metaproterenol, 10^{-6} M; 8-bromo cAMP, 10^{-7} M; glucagon, 10^{-7} M; forskolin, 10^{-7} M; dobutamine, 10^{-6} M.

Each value is expressed as the mean ± S.E.M. from three independent preparations.

Values significantly different from those for the control are indicated by [a]$P < 0.05$, [b]$P < 0.01$.

Values significantly different from those for IGF-I alone are indicated by [c]$P < 0.05$, [d]$P < 0.01$.

Values significantly different from those for IGF-II alone are indicated by [e]$P < 0.05$, [f]$P < 0.01$.

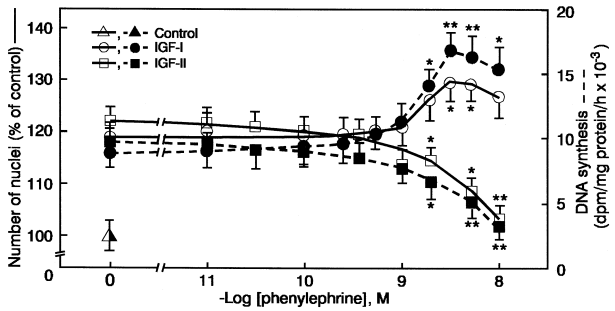

Fig. 5. Effects of phenylephrine on IGF-I- or IGF-II-stimulated hepatocyte DNA synthesis and proliferation. Hepatocytes at a density of 3.3×10^4 cells/cm^2 were plated and cultured for 3 h as described in the legend to Fig. 1. After an attachment period of 3 h (zero time), the medium was rapidly replaced with serum- and dexamethasone-free Williams' medium E and the hepatocytes were cultured with various concentrations of phenylephrine in the presence of 100 ng/ml IGF-I or 1.0 ng/ml IGF-II for an additional 4 h. Hepatocyte DNA synthesis (dotted lines) and proliferation (solid lines) were determined as described in Section 2. Data are expressed as means ± S.E.M. of three separate experiments. $*\ P<0.05$, $**\ P<0.01$ compared with respective control.

blocked the IGF-II-induced DNA synthesis and proliferation in hepatocytes cultured at 4 and 21 h (Table 1). H-89 (10^{-7} M) also reversed the inhibitory effects of both metaproterenol (10^{-6} M) and 8-br-cAMP (10^{-7} M) on DNA synthesis and proliferation in the presence of 1.0 ng/ml IGF-II in hepatocytes cultured at 4 h and 21 h. The potentiating effects of UK-14304 (10^{-5} M) on IGF-II-induced hepatocyte DNA synthesis and proliferation were blocked by metaproterenol, but not by 8-br-cAMP, suggesting that UK-14304 acts at the receptor level. Furthermore, in contrast to metaproterenol, the β_1-adrenoceptor agonist, dobutamine (10^{-6} M), did not influence IGF-I- or IGF-II-induced hepatocyte DNA and proliferation, suggesting that the β_1-adrenoceptor-mediated system is not involved in the responses.

3.6. Effects of phenylephrine on IGF-I or IGF-II-stimulated hepatocyte DNA synthesis and proliferation

α_1-Adrenoceptor agonists, such as norepinephrine or phenylephrine, modulate hepatocyte proliferation in vitro. Therefore, we examined the effects of phenylephrine on IGF-I- or IGF-II-induced hepatocyte mitogenesis after 4 h of culture. Fig. 5 shows that the ability of IGF-I to induce hepatocyte DNA synthesis and proliferation was potentiated by phenylephrine in concentrations up to 5×10^{-9} M. This effect was dose dependent and phenylephrine became slightly inhibitory at 10^{-8} M. In contrast, IGF-II-induced hepatocyte DNA synthesis and proliferation were dose dependently inhibited by phenylephrine, although phenylephrine (10^{-11} M–10^{-8} M) alone had no significant effect on hepatocyte DNA synthesis and proliferation.

Table 2
Effects of sphingosine on PMA-induced hepatocyte DNA synthesis and proliferation in the presence of IGF-I or IGF-II

Agent	DNA synthesis (dpm/mg protein per h $\times 10^{-3}$)		Number of nuclei (% of control)	
	Culture time (h)		Culture time (h)	
	4	21	4	21
Control	2.068 ± 0.280	1.695 ± 0.432	100.4 ± 3.3	99.8 ± 3.2
IGF-I	8.733 ± 1.063^{b}	5.215 ± 1.183^{a}	121.2 ± 1.8^{b}	122.8 ± 1.7^{b}
+ U73122	3.352 ± 1.107^{c}	8.132 ± 1.212^{b}	102.2 ± 2.2^{d}	103.6 ± 2.6^{d}
+ U73343	8.546 ± 1.118^{b}	5.132 ± 1.120^{a}	122.3 ± 2.3^{b}	123.0 ± 2.4^{b}
+ Phenylephrine	15.368 ± 1.411^{b,c}	13.083 ± 1.329^{b,c}	133.2 ± 3.2^{b,c}	139.6 ± 4.2^{b,c}
+ Sphingosine	8.367 ± 1.217^{b}	5.106 ± 1.122^{a}	122.0 ± 2.2^{b}	123.6 ± 2.6^{b}
+ PMA	15.736 ± 1.442^{b,c}	12.071 ± 1.177^{b,c}	136.2 ± 4.2^{b,c}	137.6 ± 4.6^{b,c}
+ PMA + sphingosine	8.231 ± 1.146^{b}	5.173 ± 1.027^{a}	125.2 ± 2.2^{b}	125.6 ± 4.6^{b}
IGF-II	10.276 ± 1.025^{b}	10.193 ± 1.030^{b}	124.3 ± 3.3^{b}	124.5 ± 3.1^{b}
+ U73122	9.654 ± 1.107^{b}	9.932 ± 1.012^{b}	122.0 ± 2.0^{b}	123.6 ± 2.1^{b}
+ U73343	9.776 ± 1.163^{b}	10.100 ± 1.131^{b}	122.4 ± 2.8^{b}	123.9 ± 2.3^{b}
+ Phenylephrine	3.371 ± 1.218^{e}	2.086 ± 1.520^{e}	105.2 ± 3.2^{e}	105.6 ± 4.4^{e}
+ Sphingosine	9.367 ± 1.217^{b}	10.006 ± 1.122^{b}	122.0 ± 2.2^{b}	123.6 ± 2.6^{b}
+ PMA	2.668 ± 0.442^{f}	3.571 ± 1.077^{e}	104.2 ± 4.2^{e}	104.6 ± 3.6^{e}
+ PMA + sphingosine	9.898 ± 0.933^{b}	9.889 ± 1.142^{b}	124.2 ± 5.2^{a}	124.5 ± 3.7^{b}

Hepatocytes were plated at a density of 3.3×10^4 cells/cm^2 and cultured for 4 and 21 h with 100 ng/ml IGF-I alone or 1 ng/ml IGF-II alone or with phenylephrine, sphingosine and PMA immediately after the medium was changed. U73122, 10^{-5} M; U73343, 10^{-5} M; phenylephrine, 3×10^{-9} M; sphingosine, 3×10^{-6} M; phorbol 12-myristate 13-acetate (PMA), 10^{-8} M.
Each value is expressed as the mean ± S.E.M. from three independent preparations.
Values significantly different from those for the control are indicated by $^{a}P<0.05$, $^{b}P<0.01$.
Values significantly different from those for IGF-I alone are indicated by $^{c}P<0.05$, $^{d}P<0.01$.
Values significantly different from those for IGF-II alone are indicated by $^{e}P<0.05$, $^{f}P<0.01$.

3.7. Effects of sphingosine on PMA-induced hepatocyte DNA synthesis and proliferation in the presence of IGF-I or IGF-II

To characterize the involvement of the phospholipase C/protein kinase C pathway in IGF-I- and IGF-II-induced hepatocyte DNA synthesis and proliferation, we investigated the effects of U-73122 (Thompson et al., 1991), a phospholipase C inhibitor, phorbol 12-myristate 13-acetate (PMA) (Castagana et al., 1982), a cell-permeable analogue of diacylglycerol, and sphingosine (Merrill et al., 1989), a specific protein kinase C inhibitor, on the responses induced by IGF-I or IGF-II. Table 2 shows that the phospholipase C inhibitor, U-73122 (10^{-5} M), significantly attenuated the action of IGF-I on hepatocyte DNA synthesis and proliferation after 4 h of culture. The inhibitory effect of IGF-I on hepatocyte DNA synthesis at 21 h of culture was partially restored by U-73122. In contrast, U-73343 (10^{-5} M), a close structural analog of U-73122 that does not inhibit phospholipase C, did not significantly affect IGF-I-induced hepatocyte DNA synthesis and proliferation. To determine whether or not diacylglycerol, a direct effector of protein kinase C, is involved in IGF-induced hepatocyte DNA synthesis and proliferation, hepatocytes were incubated with PMA (10^{-8} M) for 4 and 21 h. Although PMA alone had no significant effect on hepatocyte DNA synthesis and proliferation (data not shown), in the presence of IGF-I DNA synthesis and proliferation were potentiated by PMA after 4 h of culture and slightly reduced after 21 h of culture. The potentiating effects of PMA were significantly reversed by coincubation with the protein kinase C inhibitor, sphingosine (3×10^{-6} M), for 4 and 21 h. U-73122 (10^{-5} M) did not significantly attenuate the action of IGF-II (1.0 ng/ml) on hepatocyte DNA synthesis and proliferation during 4 h and 21 h of culture. U-73343 (10^{-5} M) did not significantly affect IGF-II-induced hepatocyte DNA synthesis and proliferation. To determine whether or not diacylglycerol is involved in IGF-II-induced hepatocyte DNA synthesis and proliferation, hepatocytes were incubated with PMA for 4 and 21 h. PMA (10^{-8} M) alone had no significant effect on these responses (data not shown) but significantly reduced the ability of IGF-II to stimulate hepatocyte DNA synthesis and proliferation during the early and late phases of culture. The inhibitory effects of PMA were sustained for 21 h but were largely reversed during 4 and 21 h of culture with sphingosine (3×10^{-6} M) in the presence of IGF-II. Similarly, to determine whether or not intracellular Ca^{2+} mobilization is involved in hepatocyte DNA synthesis and proliferation, cells were incubated with the calcium ionophore, ionomycin, for 4 and 21 h in the presence of IGF-I (100 ng/ml) or IGF-II (1.0 ng/ml). Hepatocyte DNA synthesis and proliferation were not affected by ionomycin in doses up to 10^{-5} M (data not shown).

3.8. Effects of specific inhibitors of signal transducing elements on hepatocyte DNA synthesis and proliferation-induced by IGF-I or IGF-II

We investigated whether or not the mitogenic responses of hepatocytes to 100 ng/ml IGF-I and 1.0 ng/ml IGF-II

Table 3
Effects of specific inhibitors of signal-transducing elements on hepatocyte DNA synthesis and proliferation induced by IGF-I or IGF-II

Agent	DNA synthesis (dpm/mg protein per h $\times 10^{-3}$)		Number of nuclei (% of control)	
	Culture time (h)		Culture time (h)	
	4	21	4	21
Control	2.203 ± 0.352	2.120 ± 0.290	100.5 ± 2.8	100.1 ± 3.1
IGF-I	9.886 ± 1.487^{b}	6.039 ± 1.290^{a}	120.8 ± 2.5^{b}	123.2 ± 2.0^{b}
+ Pertussis toxin	9.503 ± 0.643^{b}	5.789 ± 0.932^{a}	122.3 ± 2.1^{b}	123.9 ± 4.2^{a}
+ Genistein	2.501 ± 0.547^{d}	1.729 ± 0.632^{c}	102.3 ± 2.1^{d}	103.9 ± 4.2^{c}
+ Wortmannin	3.142 ± 0.580^{c}	1.802 ± 0.507^{c}	101.2 ± 3.5^{c}	100.6 ± 4.0^{d}
+ PD98059	2.444 ± 0.642^{c}	2.409 ± 0.203^{c}	100.8 ± 3.3^{d}	101.1 ± 3.2^{d}
+ Rapamycin	1.515 ± 0.250^{d}	0.231 ± 0.115^{c}	100.6 ± 4.2^{c}	101.7 ± 4.3^{c}
IGF-II	10.273 ± 1.662^{b}	10.039 ± 1.246^{b}	122.4 ± 3.2^{b}	123.5 ± 2.0^{b}
+ Pertussis toxin	2.106 ± 0.342^{e}	2.389 ± 0.132^{f}	102.3 ± 2.1^{f}	103.9 ± 2.2^{f}
+ Genistein	10.503 ± 1.527^{b}	9.709 ± 0.992^{b}	123.3 ± 4.0^{b}	124.9 ± 2.6^{b}
+ Wortmannin	10.421 ± 0.984^{b}	10.033 ± 0.906^{b}	120.2 ± 3.5^{a}	122.3 ± 5.0^{a}
+ PD98059	2.402 ± 0.786^{e}	4.498 ± 1.337^{e}	105.1 ± 2.9^{e}	103.1 ± 3.6^{f}
+ Rapamycin	3.519 ± 0.353^{e}	1.383 ± 0.103^{f}	108.3 ± 3.1^{e}	109.7 ± 4.2^{e}

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 inhibitors of signal-transducing elements were added with 100 ng/ml IGF-I or 1 ng/ml IGF-II immediately after the medium was changed and cells were cultured for a further 4 and 21 h. Genistein, 5×10^{-6} M; pertussis toxin, 100 ng/ml; wortmannin, 10^{-7} M; PD98059, 5×10^{-5} M; rapamycin, 10 ng/ml.
Each value is expressed as the mean ± S.E.M. from three independent preparations.
Values significantly different from those for the control are indicated by $^{a}P < 0.05$, $^{b}P < 0.01$.
Values significantly different from those for IGF-I alone are indicated by $^{c}P < 0.05$, $^{d}P < 0.01$.
Values significantly different from those for IGF-II alone are indicated by $^{e}P < 0.05$, $^{f}P < 0.01$.

are mediated by signal transducers, such as inhibitory G-protein (G_i), receptor tyrosine kinase, phosphatidylinositol 3-kinase (PI(3)K), mitogen-activated protein (MAP) kinase kinase, and ribosomal protein p70 S6 kinase, by using specific inhibitors of these signal transducers (Table 3). The effects of IGF-I on hepatocyte DNA synthesis and proliferation were not affected by the specific Gi-protein inhibitor, pertussis toxin (100 ng/ml; Katada and Ui, 1982). Genistein (5×10^{-6} M), a specific inhibitor of receptor tyrosine kinase (Akiyama et al., 1987), wortmannin (10^{-7} M), a specific inhibitor of PI(3)K (Baggiolini et al., 1987), PD98059 (5×10^{-5} M), a specific inhibitor of MAP kinase kinase (Alessi et al., 1995), and rapamycin (10 ng/ml), a specific inhibitor of p70 S6 kinase inhibitor (Chung et al., 1992; Price et al., 1992), alone had no effect on hepatocyte DNA synthesis and proliferation, but all these agents almost completely blocked the stimulation induced by IGF-I during the early and late phases of culture. In contrast, the effects of IGF-II (1.0 ng/ml) on hepatocyte DNA synthesis and proliferation were completely abolished by pertussis toxin (100 ng/ml). Genistein (5×10^{-6} M) or wortmannin (10^{-7} M) did not inhibit significantly IGF-II-induced hepatocyte DNA synthesis and proliferation. The requirement for the MAP kinase cascade in hepatocyte DNA synthesis and proliferation was examined by using PD98059 (5×10^{-5} M), an inhibitor of MAP kinase kinase. PD98059 attenuated IGF-II-induced hepatocyte DNA synthesis and proliferation, suggesting that MAP kinase kinase mediates the responses. Rapamycin (10 ng/ml) also blocked the hepatocyte DNA synthesis and proliferation induced by 1.0 ng/ml IGF-II.

4. Discussion

As shown in Fig. 1, both IGF-I and IGF-II rapidly stimulated hepatocyte DNA synthesis and proliferation as compared with the results cited in previous reports (Nakamura et al., 1983a; Refsnes et al., 1992). This difference in the time course between our results and those of others is thought to be due to the concentrations of dexamethasone in the culture medium, since hepatocyte DNA synthesis and proliferation induced by IGF-I or IGF-II were reduced in a dose- and time-dependent manner in response to increasing concentrations of dexamethasone (10^{-9}–10^{-7} M) in the culture medium. For instance, IGF-I (100 ng/ml) or IGF-II (1 ng/ml)-induced hepatocyte DNA synthesis and proliferation decreased by approximately 50% after 4 h of culture when a relatively large amount of dexamethasone (10^{-9} M) was added during the 3-h attachment period. The inhibitory effects of dexamethasone on IGF-I- or IGF-II-stimulated hepatocyte DNA synthesis and proliferation were partially restored after culture without dexamethasone for a further 17 h (Kimura and Ogihara, unpublished observation). These data are in accordance with previously published observations for primary cultured hepatocytes exposed to 5 ng/ml HGF (Kimura and Ogihara, 1997c).

As shown in Fig. 2, the pronounced differences between the potencies of and maximal responses to IGF-I and IGF-II may be due, in part, to the expression of some members of the IGF-binding protein family. The IGF-binding protein family serves as a reservoir of IGFs and is known to regulate the access of IGFs to IGF receptors in some cell types (Czech, 1989; Humbel, 1990). Hepatocytes are known to secrete IGF-I and its modulatory binding proteins. It is, therefore, important to identify the forms of IGF-binding protein secreted by hepatocytes and to determine the mode of action of each. However, their precise biological actions are poorly defined in this culture system, and the reason for this pronounced difference between the potencies of and maximal responses to IGF-I and IGF-II requires further study. In addition, the effects of IGF-I on hepatocyte DNA synthesis and proliferation were highly dependent on cell density, while those of IGF-II were not (Fig. 3A and B), suggesting a separate signalling pathway for hepatocyte proliferation. The underlying mechanisms of these phenomena remain obscure. However, cell-to-cell contact (Nakamura et al., 1983b) and/or secretion of some kinds of autocrine factors by hepatocytes may regulate the hepatocyte mitogenesis induced by each IGF peptide (Nakamura et al., 1983a).

We have shown that α- and β-adrenoceptor agonists significantly modulate hepatocyte DNA synthesis and proliferation in the presence of growth factors such as EGF (Kimura and Ogihara, 1997a), insulin (Kimura and Ogihara, 1997b), HGF (Kimura and Ogihara, 1997c), and PDGF (Kimura and Ogihara, 1998). These adrenoceptor agonists alter cellular cAMP levels. However, the role of cAMP in cell growth is controversial (Rosengurt, 1982; Bronstad et al., 1983; Refsnes et al., 1983; Refsnes et al., 1992). We therefore examined the role of cAMP in hepatocyte mitogenesis induced by IGF-I and IGF-II. In accordance with reported results, we found that metaproterenol and other cAMP-elevating agents (8-br-cAMP, glucagon and forskolin) potentiated IGF-I-induced hepatocyte DNA synthesis by a parallel stimulation of proliferation (Fig. 4). In contrast, cAMP-elevating agents almost completely blocked IGF-II-induced DNA synthesis and proliferation in hepatocytes cultured for 4 and 21 h (Table 1). H-89, a specific inhibitor of cAMP-dependent protein kinase, reversed both effects of the cAMP-elevating agents, confirming the involvement of protein kinase A. However, why activation of the same cAMP/protein kinase A cascade causes contrasting biological effects remains unknown. Understanding the molecular mechanisms whereby cAMP stimulates the effects of IGF-I but inhibits those of IGF-II, and the point of convergence between IGFs and the protein kinase A pathways remains to be elucidated.

Moreover, we found that hepatocyte DNA synthesis induced by 1.0 ng/ml IGF-II alone was potentiated dose

dependently by UK-14304 (Fig. 4B), whereas that induced by 100 ng/ml IGF-I was not affected by this α_2-adrenoceptor agonist (Fig. 4B). The effects of UK-14304 on IGF-I- or IGF-II-induced hepatocyte DNA synthesis closely correlated with the altered hepatocyte proliferation. The results suggest that α_2-adrenoceptor-Gi protein-mediated signals, which may decrease cellular cAMP levels, interact with the IGF-II signal transduction pathways.

As shown in Fig. 5 and Table 2, the effects of phenylephrine, U-73122 and PMA on IGF-I-induced hepatocyte DNA synthesis and proliferation are in marked contrast to the effects on the IGF-II-induced responses. However, why activation of the diacylglycerol/protein kinase C cascade causes different biological effects remains unknown. Tyrosine kinase receptors, such as EGF, PDGF and IGF-I, generate the second messengers phosphatidyl inositol (1, 4, 5) trisphosphate (IP_3) and diacylglycerol by direct interaction with phospholipase C-γ to stimulate hepatocyte growth and proliferation (Berridge, 1993). The α_1-adrenoceptor agonist, phenylephrine, exerts its action by stimulating phospholipase C-β via Gq-protein. The activated phospholipase C-β also catalyzes the hydrolysis of phosphatidylinositol 4, 5-bisphosphate to generate IP_3 and DG. IP_3 leads to mobilization of calcium ions from intracellular stores, whereas diacylglycerol activates several protein kinase C isozymes. Therefore, diacylglycerol at various cellular sites may activate different protein kinase C isozymes to mediate the contrasting effects of IGF-I or IGF-II on hepatocyte DNA synthesis and proliferation (Nishizuka, 1986; Valverde et al., 1996). Our results provide evidence of positive cross-talk between the protein kinase C and IGF-I pathways and negative cross-talk between the protein kinase C and IGF-II pathways. The point of convergence between IGFs and the protein kinase C pathways remains to be further explored. In addition, DNA synthesis was significantly reduced by IGF-I after 21 h of culture (Fig. 1 and Table 2). The mechanism underlying the reduction of hepatocyte DNA synthesis may be related to down-regulation (or desensitization) of protein kinase C activity, since this reduction was significantly reversed by coincubation with the protein kinase C inhibitor, sphingosine (3×10^{-6} M; Table 2). This agonist-induced desensitization of protein kinase C activity during long-term culture is well documented (Grove and Mastro, 1988).

Other specific inhibitors of the intracellular signalling cascade are also useful probes with which to characterize the target proteins involved in the activation of DNA synthesis and proliferation induced by IGFs in cultured adult rat hepatocytes. Hepatocyte DNA synthesis and proliferation induced by IGF-I were significantly blocked by genistein, wortmannin, and rapamycin, suggesting that hepatocyte mitogenesis is stimulated through receptors that are associated with tyrosine kinase, PI(3)K (Myers et al., 1993), and p70 S6K. In contrast, since hepatocyte DNA synthesis and proliferation induced by IGF-II were not influenced by genistein and wortmannin, tyrosine kinase and PI(3)K are not involved in these responses. Prior incubation with the Gi-protein inhibitor, pertussis toxin, completely abolished only the IGF-II effects on hepatocyte DNA synthesis and proliferation, suggesting that IGF-II receptors, but not IGF-I receptors, are linked to the Gi protein. These results are consistent with the reported finding that IGF-II-induced proliferation in cultured BALB/c 3T3 cells is mediated through Gi-protein (Nishimoto et al., 1987).

MAP kinase is commonly activated by several extracellular stimuli and is supposed to play a key role in various intracellular signal transduction pathways (Davis, 1993). Using PD98059, which specifically blocks the activation of MAP kinase kinase-1 (Alessi et al., 1995), we demonstrated that these signal transducers are involved in the hepatocyte DNA synthesis and proliferation induced by IGF-I or IGF-II (Table 3). All the signal transducing elements described above play a critical role in stimulating hepatocyte DNA synthesis and proliferation, although the cascades of sequential phosphorylation events are not yet completely defined.

In conclusion, although the physiological importance of IGF-I and IGF-II cannot be properly gauged from this in-vitro study alone, these results demonstrate for the first time that IGF-I and IGF-II can rapidly induce DNA synthesis and proliferation in primary cultures of adult rat hepatocytes. The proliferative effects of IGF-I and IGF-II are apparently caused by different mechanisms. The IGF-I mechanism was cell density-dependent and was stimulated by cAMP-elevating agents, an α_1-adrenoceptor agonist and PMA. It was mediated by tyrosine kinase, phospholipase C, PI(3)K and p70 S6K. In contrast, the mechanism of IGF-II was independent of plating density and was inhibited by cAMP-elevating agents, an α_1-adrenoceptor agonist, and PMA. It was mediated by Gi protein, MAP kinase and p70 S6K. Thus, we provide evidence of differences in the signal transducing elements that control the hepatocyte DNA synthesis and proliferation induced by IGF-I or IGF-II. These signalling pathways may ultimately provide strategies for the selective activation of hepatocyte proliferation in vivo.

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