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Hepatocyte growth factor (HGF) mediates the sustained formation of 1,2-diacylglycerol via phosphatidylcholine–phospholipase C in cultured rat hepatocytes

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The addition of hepatocyte growth factor (HGF) to rat hepatocytes in primary culture resulted in the formation of inositol 1,4,5-trisphosphate ($Ins(1,4,5)P_3$) and 1,2-diacylglycerol (DG) by a phosphoinositide-specific phospholipase C (PI-PLC). DG showed a biphasic increase; the first phase, corresponding with the peak of $Ins(1,4,5)P_3$ and a second larger and prolonged phase. The HGF stimulates the phosphatidylcholine (PC)-derived prolonged DG formation by a phospholipase C pathway (PC-PLC) but not by a phospholipase D pathway. HGF also was found to elicit [Ca^{2+}] oscillations which may be associated with the prolonged DG production from PC via the PC-PLC phospholipase C pathway.

Hepatocyte growth factor (HGF); Sustained DG formation; Phosphatidylcholine-phospholipase C; Ca^{2+} oscillation

1. INTRODUCTION

Hepatocyte growth factor (HGF) was first detected as a potent mitogen for mature hepatocytes in primary culture in serum of partially hepatectomized rats [1] and in rat platelets [2]. This heterodimer growth factor is regarded as a trigger for liver regeneration in vivo after liver injury [3]. The genes encoding human [4,5] and rat [6] HGF have been cloned and sequenced. HGF mRNA is expressed not only in liver non-parenchymal cells but also in various rat tissues such as kidney, heart, lung and brain [6]. HGF also stimulates DNA synthesis and the growth of many types of epithelial cells such as epidermal keratinocytes [7], renal tubular cells [8] and melanocytes [9]. More recently, HGF was found to be structurally and functionally identical to scatter factor [10].

The receptor for HGF has been identified to be the c-*met* proto-oncogene product [11,12]. Although the precise signaling mechanism following the activation of HGF receptor is not clear, it has recently been suggested that HGF induces tyrosine phosphorylation of target cell proteins. Accumulating evidence indicates that one of the biochemical events implicated in mitogenic signal transduction is elevation of 1,2-diacylglycerol (DG). The main source of DG was thought to be phosphoinositides, but it has recently become clear that phosphatidylcholine (PK) serves as an alternative source for DG production (for review see [13,14]. PC can be hydrolyzed by either phospholipase C (PC-PLC), distinct from PI-PLC acting on phosphoinositides, or phospholipase D (PC-PLD) to yield DG and phosphatidic acid (PA), respectively. In order to understand the HGF-mediated signaling pathway, we examined the production of DG in cultured rat hepatocytes stimulated with HGF. The results obtained here indicate that HGF stimulates a prolonged DG production via the direct PC-PLC pathway rather than the indirect PC-PLD pathway and that the activation of PC-PLC is somehow associated with $[Ca^{2+}]_i$ oscillations.

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2. EXPERIMENTAL

2.1. *Materials*

[^{3}H]Arachidonic acid (207 Ci/mmol) and the $Ins(1,4,5)P_3$ assay system were obtained from Amersham. [^{3}H]Myristic acid was from New England Nuclear. Methyl[^{3}H]choline chloride (85 Ci/mmol) was from American Radiolabeled Chemicals. Fura 2/AM was purchased from Dojin Laboratories (Kumamoto, Japan). Cell culture reagents were obtained as described previously [16]. All other chemicals were of reagent grade.

2.2. *Isolation and monolayer culture of rat hepatocytes*

Hepatocytes were isolated from adult male Wistar rats (about 200 g) by perfusion of the liver in situ with collagenase as previously described [1,15]. The isolated cells (5.0×10^4 cells/cm^2) were first cultured as monolayers in plastic culture dishes which had been coated with rat tail collagen in Williams medium E supplemented with 5% calf serum, 10^{-9} M dexamethasone and 10^{-9} M insulin.

2.3. *Assay of DNA synthesis*

DNA synthesis was assayed as described previously [16].

2.4. *Radiolabeled lipid and choline metabolite analyses*

For the studies of fatty acid-labeled lipid metabolism, hepatocytes were labeled with [^{3}H]myristic acid (0.5 μCi/ml) or methyl[^{3}H]choline (1 μCi/ml) for 20 h in medium. Incubations in the presence or absence of 20 ng/ml of HGF were stopped by removing the medium and immediately adding 1 ml of ice-cold phosphate-buffered saline/methanol mixture (2:5, v/v). Total cellular lipids were extracted as described [17]. The mass content of Ins(1,4,5)P_3 was quantified as described previously [18]. For analysis of water-soluble choline metabolites, the aqueous phase of the chloroform/methanol extract was dried and resuspended in 50% (v/v) ethanol. A portion of each sample was spotted on a LK6D plate and then developed with 0.5% NaCl/methanol/conc. NH_3 (50:50:1, v/v) [19].

2.5. *Measurement of $[Ca^{2+}]_i$ in single cells*

The fluorescence image (excitation at 340 and 360 nm, emission at 500 nm) analysis and calibration of Ca^{2+} concentrations were carried out as described previously [18].

3. RESULTS

HGF stimulated DNA synthesis of rat mature hepatocytes in primary culture in a concentration-dependent manner (Fig. 1). The maximal stimulation of DNA synthesis was obtained at 20 ng/ml HGF. EGF at the same concentration (20 ng/ml) induced the increase in DNA synthesis but the stimulatory potency was much lower compared with HGF. The effect of EGF on HGF-induced DNA synthesis was nearly additive.

Stimulation by HGF (20 ng/ml) of hepatocytes labeled with [^{3}H]myristic acid, in which the major labeled phospholipid was PC (81.7 ± 1.3%; n=6), caused a biphasic accumulation of DG (Fig. 2B). The first phase showed a small transient peak within 20 s of stimulation, whereas the second phase was a larger and prolonged elevation. Furthermore, HGF elicited a rapid and transient production of Ins(1,4,5)P_3, coincident with the first phase of DG formation (Fig. 2A). Although the radioactivities in other phospholipids such as PI, PE, and PS were not significantly changed, a decrease in [^{3}H]PC was distinct in response to HGF (Fig. 3), suggesting that the second prolonged phase of DG was derived from PC. The level of [^{3}H]triacylglycerol was increased after HGF-stimulation (resting, 20,260 ± 352 dpm/10^5 cells; 10-min HGF-treated, 24,832 ± 681 dpm/10^5 cells).

The analysis of water-soluble choline metabolites revealed that [^{3}H]phosphocholine was increased in a time-dependent manner (Fig. 4). In contrast, the level of [^{3}H]choline was not significantly changed during the period tested (10 min). These data indicate that the prolonged DG formation is mediated by the PC-PLC pathway.

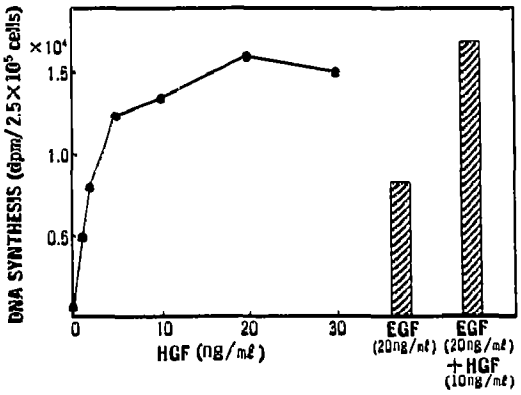

Fig. 1. HGF-induced DNA synthesis at various concentrations. Cells exposed to HGF at indicated concentrations were labeled with [^{3}H]thymidine and analysed for DNA synthesis. EGF (20 ng/ml) was added alone or in combination with HGF (10 ng/ml). The results are the means of two experiments performed in duplicate.

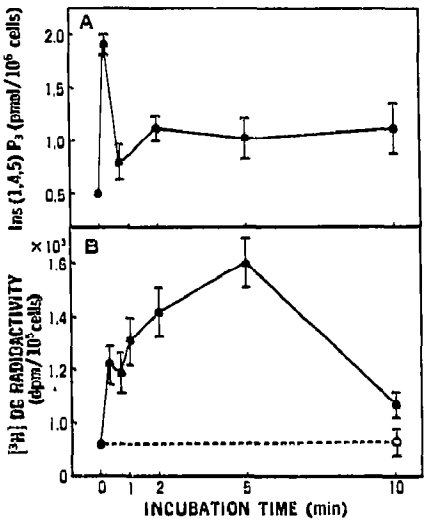

Fig. 2. Time-courses of HGF-stimulated formation of DG and Ins(1,4,5)P_3 in cultured rat hepatocytes. (A) Mass content of Ins(1,4,5)P_3 was determined at indicated times using the assay system. (B) After labeling for 20 h with [^{3}H]myristic acid, cells were exposed to 20 ng/ml of HGF for the times indicated. The radioactivity in unstimulated control (921.5 ± 23.7 dpm) was designated as 100%. Each point is the mean ± S.D. from four experiments performed in duplicate.

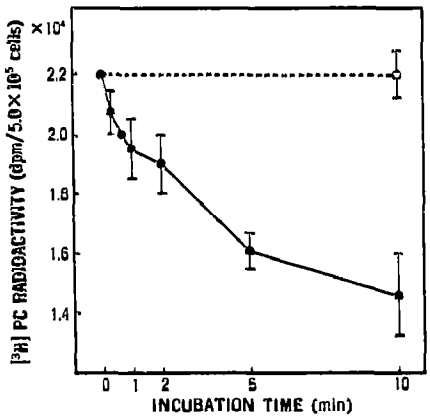

Fig. 3. Time-course of HGF-induced radioactivity change in phosphatidylcholine (PC) in [^{3}H]myristic acid-labeled hepatocytes. Cells labeled with [^{3}H]myristic acid for 20 h were stimulated with 20 ng/ml of HGF. Each point is the mean ± S.D. from four experiments performed in duplicate.

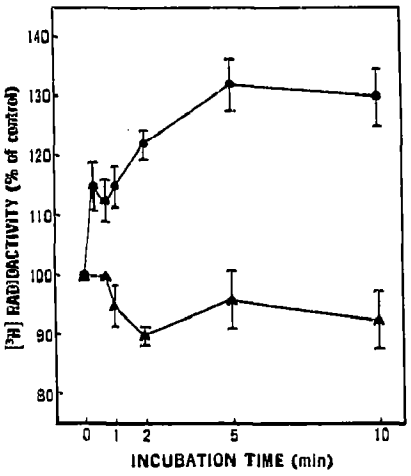

Fig. 4. Time-courses of HGF-induced radioactivity changes in phosphocholine and choline in [^{3}H]choline-labeled hepatocytes. Hepatocytes were labeled for 20 h with [^{3}H]choline, washed and stimulated with 20 ng/ml of HGF. Lipids and water-soluble metabolites were separated and analyzed. The radioactivity in unstimulated control (phosphocholine, 21,452.1 ± 567 dpm; choline 1,145.2 ± 74.9 dpm) was designated as 100%: (●) phosphorylcholine; (▲) choline. Each point is the mean ± S.D. from four experiments performed in duplicate.

To more carefully assess the action of the direct formation of DG by PC-PLC, the other DG pathway by PLD was examined. Propranolol, commonly used as an inhibitor of PA phosphohydrolase [20], did not affect DG production in response to HGF. Furthermore, in the presence of ethanol, PLD catalyzes the transphosphatidylation of PC to yield a specific phospholipid, phosphatidylethanol (PEt) which is a useful marker for PLD activity [21]. However, activation of hepatocytes with HGF in the presence of ethanol (0.5, 1.0, 1.5, 2.5%) failed to increase the level of PEt. Such ethanol treatment had no effect on HGF-mediated production of DG. Taken together, these results again indicate that HGF stimulates hydrolysis of PC by the action of PLC and not PLD, resulting in the slow prolonged DG accumulation. It was also found that HGF evoked oscillatory $[Ca^{2+}]_i$ changes in fura 2-loaded hepatocytes (approx. over 60% of total cells) (data not shown). Addition of EGTA (2.4 mM) resulted in the inhibition of $[Ca^{2+}]_i$ oscillations and also the prolonged DG production in response to HGF.

4. DISCUSSION

Accumulating evidence suggests that DG plays an important role in the mitogenic signal transduction induced by various growth factors [22–28]. The sustained accumulation of DG has been reported in response to mitogenic stimuli such as EGF, PDGF and α-thrombin [25]. DG activates PKC [27] and other, as yet unidentified, signaling pathway(s) [28,29]. Previous studies suggested that agonist-induced formation of DG resulted from hydrolysis of phosphoinositides, especially PIP_2 [30]. Recent evidence indicates that PC provides an alternative source for DG production. Agonist-induced hydrolysis of PC is attributed to the main two pathways mediated via PLC and/or PLD. Both activities have been reported to be present in rat hepatocytes [31–33]. In the present study, we have demonstrated that HGF stimulated PIP_2 hydrolysis and subsequent prolonged DG formation in rat hepatocytes, and also that the prolonged DG formation induced in response to HGF is principally derived from PC by the action of PLC. The PLC-mediated PC hydrolysis was thought to be stimulated by the increased $[Ca^{2+}]_i$ [35] or a GTP-binding protein [32], especially *ras* oncogene product, *ras* p21 [27]. We have recently suggested evidence that in *ras*-transfected fibroblasts the oscillations of $[Ca^{2+}]_i$ are somehow associated with the sustained DG formation via the PC-PLC pathway. Therefore, the data obtained here that depletion of extracellular Ca^{2+} inhibits both prolonged DG production and $[Ca^{2+}]_i$ oscillations elicited by HGF, also support the idea of a possible association of the PC-PLC pathway with $[Ca^{2+}]_i$ oscillations.

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