Parathyroid Hormone Co-stimulation of Hepatocyte Proliferation is Sensitive to Protein Kinase A and Calcium Channel Inhibitors

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Parathyroid hormone (PTH) mobilises calcium in the hepatocyte, an effect which is abolished by verapamil and staurosporine. In our study parathyroid hormone was shown to act additively to dHGF in inducing hepatocyte DNA synthesis. It is also shown that PTH induced the production of inositol 1,4,5 trisphosphate (IP₃) and c-fos expression at early times in culture. Co-incubation of PTH and dHGF with a c-fos antisense oligodeoxynucleotide inhibited hepatocyte DNA synthesis, indicating that the additive effect of PTH is correlated with the induction of c-fos. H-89, a PKA specific inhibitor, inhibited the PTH effect on IP₃ production as well as the PTH effect on hepatocyte DNA synthesis. Verapamil and staurosporine also inhibited the PTH effect in dHGF-induced DNA synthesis. Therefore it is suggested that PKA mediated at a great extent the co-stimulatory effects of PTH on hepatocyte proliferation via IP₃ production.

KEY WORDS: dHGF; hepatocyte; protein kinase A; calcium.

INTRODUCTION

It is known that liver is a target tissue for action of the parathyroid hormone (PTH) (1–2). PTH is mobilising calcium in the hepatocyte and this effect has been observed with both PTH (1) and its amino-terminal fragment and was shown to be a receptor-mediated effect (3). The calcium-“feeding” effect of PTH is blocked by PTH-antagonist as well as by verapamil and nifedipine (3). PTH was also shown to induce c-fos expression in osteoblastic osteosarcoma cells (4). Both cAMP and phosphoinositide-dependent signalling cascades are activated by PTH through interactions with separate G proteins. It has also been reported that activation of cAMP-dependent protein kinase (PKA) was correlated with the phosphoinositide (PI) cascade in hepatocytes (5). Primary hepatocytes undergo DNA synthesis in vitro only if stimulated with hepatocyte-specific growth factors.
such as Hepatocyte Growth Factor (HGF), Transforming Growth Factor-α (TGF-α) and Epidermal Growth Factor (EGF) (6). It has been suggested that hepatocytes become susceptible to the action of growth factors once they have exited the resting state (G₀) and progress through the G₁ phase of the cell cycle (7). Such signals rendering the hepatocytes susceptible to the growth-factor actions may be some components of the extracellular matrix (ECM) or products of genes activated immediately after tissue injury or experimental tissue dispersion (perfusion), such as the c-myc product (8–9). Calcium seemed to play a regulatory role in the process of liver regeneration, as parathyroidectomy abolished DNA synthesis induced by partial hepatectomy (10–11). We have studied the effects of the parathyroid hormone in regulating the dHGF-induced hepatocyte DNA synthesis. PTH caused an early induction in hepatocyte c-fos expression and inclusion of antisense c-fos oligodeoxynucleotides (ODNs) in culture inhibited the PTH/dHGF-induced hepatocyte DNA synthesis. Furthermore, the activity of the PTH in co-operating with dHGF to induce hepatocyte DNA synthesis seemed to be mediated through activation of PKA and subsequent formation of IP₃.

MATERIALS AND METHODS

Isolation and Culture of Primary Rat Hepatocytes

Hepatocytes from young adult rats (120–140 g) were prepared using the two-step collagenase perfusion technique (12). The purified hepatocytes were plated onto collagen-coated 60 mm dishes at a density of 5 × 10⁵ cells/3 ml of Dulbecco’s Modified Eagle’s medium (DMEM) with additions reported elsewhere (13). Hepatocytes were allowed to attach and then fed with fresh medium containing 0.5 μg/ml of insulin and 50 ng/dish of dHGF, where appropriate. The cells were incubated in a humidified 5% CO₂/95% air atmosphere and the medium was thereafter changed every 24 hours.

DNA Synthesis in Hepatocyte Cultures

[methyl-³H]Thymidine (5 μCi/dish sp.activity > 85 Ci/mmol) was added to the cultures for 2 hours. DNA and radioactivity were estimated as previously described (13, 14).

RNA Isolation and Hybridisation Conditions

Total RNA from cultured or freshly isolated hepatocytes was isolated by using the guanidinium thiocyanate-phenol method (15). RNA was electrophoresed on an 1% agarose/1 × MOPS gel and transferred onto Hybond-N membrane (Amersham Int.) [α³²P]CTP-labelled human c-fos fragment (1.2 Kb) was used and the membranes hybridised for 18 hours at 68°C and subsequently washed with 0.2% SSC, 0.1% SDS. The membranes were then exposed to Kodak XAR-2
Western Blotting Analysis of Hepatocyte Proteins

Total hepatocyte protein lysates were made by resuspending the cells (from 2–3 dishes) in lysis buffer containing 20 mM HEPES, 5 mM KCl, 5 mM MgCl₂, 0.5% Triton X-100, 0.1% sodium deoxycholate and 1 mM Phenyl Methyl Sulphonyl Fluoride (PMSF). Hepatocyte proteins (25 μg/lane) were separated onto a 15% Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) and then transferred onto Immobilon membrane using a BioRad protein-transfer apparatus. Membranes were blocked with Phosphate Buffered Saline (PBS)/0.1% Tween 20 containing 5% Bovine Serum Albumin (BSA) for 30 minutes, washed three times with PBS-Tween 20 at 0.1% and then incubated with a *fos* polyclonal antibody (anti-*fos*, Dianova). Anti rabbit-peroxidase-labelled secondary antibody (Dianova) was then added and reactive proteins were revealed using a commercially available enhanced chemoluminscence (ECL) Kit (Amersham Int.).

Assay of Hepatocyte IP₃ Levels

Hepatocyte IP₃ levels were assayed as previously described using a protein binding assay kit of Amersham Int. (25).

RESULTS AND DISCUSSION

In primary hepatocyte cultures, PTH was added at concentrations ranging between 10⁶–10⁻¹⁰ M. PTH was present throughout the culture time and at 24–96 hours ³H–TdR incorporation into hepatocyte DNA was measured (Fig. 1). PTH at 10⁻⁷ M induced a detectable level of thymidine incorporation observed at 72–96 hours (Fig. 1,b), however this effect was far below that observed using dHGF (a deleted form of the Hepatocyte Growth Factor, sharing the same mitogenic properties with HGF) (16). dHGF induced a marked increase in hepatocyte DNA synthesis observed between 72 and 96-hour culture (Fig. 1,c). Co-addition of PTH (10⁻⁷ M) and dHGF (50 ng/dish) in the cultures resulted in over-stimulation of thymidine incorporation at 72 and 96 hours (Fig. 1,d). The *c-fos* oncogene is involved in the regulation of cellular proliferation and in hepatocytes was found to be induced early after growth-factor stimulation (17). Neonatal hepatocytes stimulated by HGF displayed increased *c-fos* and *c-myc* expression (18). We detected a marked increase in *c-fos* expression in pTH/dHGF-stimulated hepatocyte cultures, 3 hours after the addition of the factors (Fig. 2A). dHGF also increased *c-fos* expression, however its levels were lower compared to those shown after PTH/dHGF addition. *C-fos* protein was also shown to be induced at times similar to the *c-fos* RNA expression (Fig. 2B).
In studies performed in our laboratory, the expression of c-fos was detectable also at later times in culture (not shown). More specifically we observed a stable but low expression of both the c-fos mRNA and the fos protein from 24 and for up to 96 hours in culture. This may imply that the role of c-fos as that of c-myc, is perhaps extended far below the initiation of cell proliferation in hepatocytes (19, 20).

We examined the potential of the antisense phosphorothioate c-fos ODN (5'-CGG GAG GAT GAC GCC TCG-3') (4) and of the control phosphorothioate ODN (5'GGC CAT GGC GCC TAT GAT-3') to modify the PTH/dHGF-
induced hepatocyte DNA synthesis (Fig. 3). In control studies, the antisense ODN was shown to inhibit fos protein synthesis observed at 12 and 24 hours (not shown). Pre-addition of the control and the antisense ODNs for 2 hours before the addition of dHGF resulted in no significant inhibition of hepatocyte DNA synthesis (Fig. 3: 1–3). However, under the same conditions (2-hour pre-addition), in cultures treated afterwards dHGF+PTH, the antisense ODN induced a marked decrease (approximately 65%) in DNA synthesis (Fig. 3). These data may suggest that the additive effect of the PTH on hepatocyte DNA synthesis is due to its effect in inducing c-fos.

In this view, the PTH-induced upregulation of c-fos and the subsequent transactivation of a battery of genes, might enable the hepatocyte to traverse from G0 to G1, thus becoming sensitive to the activities of various trophic factors. Inclusion of the antisense ODNs in the culture only for 5 hours, also resulted in an inhibition of PTH/dHGF-induced hepatocyte DNA synthesis (Fig. 3: 7–8).

PTH was shown to activate protein kinase C in a variety of cells such as osteoblasts (21) and renal cells (22) however no direct data is available on hepatocytes. The mechanism which PKC employed to induce a rise in hepatocyte calcium is still unclear (23). However in our studies verapamil and staurosporine a calcium channel blocker and an inhibitor of PKC respectively, induced a significant decrease in dHGF and dHGF/PTH-induced hepatocyte DNA synthesis. It is to be noted that staurosporine effect on dHGF/PTH-induced DNA synthesis was inhibitory at approximately 60% of the control value (Fig. 4: 2,5). Verapamil inhibited hepatocyte proliferation in both combinations of the growth inducers (Fig. 4: 3,6). It has been reported that verapamil causes a marked inhibition in the growth factor-induced hepatocyte DNA synthesis through a mechanism involving also, down-regulation of the c-myc gene (24).

We then explored the effect of PTH or dHGF alone or in combination of both dHGF and PTH on hepatocyte IP3 levels (Fig. 5A–C). Basal levels of IP3 in
Fig. 4. Effects of verapamil and staurosporine on PTH/dHGF-induced hepatocyte DNA synthesis. Verapamil (3,6) (20 μM) or staurosporine (2,5) (10^{-7} M) were added in dHGF-treated (1-3) or dHGF/PTH-treated (4-6) hepatocyte cultures. Control values for DNA synthesis estimated at 72 hours are given in (1) and (4). Bars represent means of values ± S.E.M. as in Fig. 1.

Fig. 5. Effects of dHGF, PTH and H89 on IP3 levels and on hepatocyte DNA synthesis. (A): IP3 levels in hepatocytes treated with dHGF (closed circles), with dHGF + H89 (10^{-7} M) (open circles) or with dHGF + H89 (10^{-5} M) (triangles), were assayed as described in the methods section. (B, C): (B): IP3 levels in hepatocytes treated with PTH (closed circles), with PTH + H89 (10^{-7} M) (open circles) or with PTH + H89 (10^{-5} M) (triangles). (C): Both factors (dHGF + PTH) were added and IP3 levels were assayed without (closed circles) or with the presence of H89 at two different concentrations (open circles, triangles) as shown in (B). (D): DNA synthesis measured at 72 hours in hepatocytes treated with dHGF (open circles) or with PTH + dHGF (triangles) in the presence of H89 at varying concentrations.
PTH and dHGF co-stimulate growth hepatocytes were below 0.3 pmol/10^6 cells at all times (not shown). IP3 levels were significantly increased under all conditions used (dHGF, PTH) although the combination of the factors has induced a more pronounced increase at 110 sec. time point. It has been shown that dHGF increases the IP3 levels in hepatocyte by activating a phosphoinositide-specific phospholipase C (PI-PLC) (25). PTH was also shown to increase IP3 levels in chondrocytes (26). Therefore we used the H-89 a specific inhibitor of protein kinase A (PKA) (27) in order to study its effects on the dHGF or the dHGF/PTH-induced hepatocyte DNA synthesis (Fig. 5D). Increasing concentrations of H-89 significantly decreased the incorporation of 3H-TdR into hepatocyte DNA as shown in Fig. 5D at 72 hours and also observed throughout the culture time (not shown). It has been reported that PTH stimulates a G protein adenylate cyclase-cAMP pathway which subsequently stimulates calcium influx sensitive to verapamil activity in hepatocytes (28). By using the H-89 inhibitor, we were able to observe a dramatic decrease in IP3 levels induced by PTH alone, thus confirming that in hepatocytes IP3 production is mediated through activation of PKA, in contrast to other observations in chondrocytes (26). From these data it is suggested that co-stimulation of hepatocyte DNA synthesis by PTH is partly mediated by an IP3 and a calcium rise, with subsequent activation of PKA. In contrast, cultures stimulated with dHGF and treated with H-89 (10^-5 M) have shown a less remarkable decrease in IP3 levels (43%) at the 110 sec. time point. These results further support the evidence that activation of a PLC-dependent pathway is the one which is primarily operating in increasing hepatocyte IP3 levels after dHGF stimulation (25).

REFERENCES