Phorbol esters, bombesin and insulin elicit differential responses on the 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase system in primary cultures of foetal and adult rat hepatocytes

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The effects of 4β-phorbol 12-myristate 13-acetate (PMA), bombesin and insulin on 6-phosphofructo-2-kinase (PFK-2) activity, on fructose 2,6-bisphosphate concentration and on the phosphorylation state of PFK-2 were investigated in primary cultures of hepatocytes from foetal and adult rats. Bombesin stimulated PFK-2 activity and increased hexose phosphate (glucose 6-phosphate and fructose 6-phosphate) and fructose 2,6-bisphosphate content in hepatocytes both in the foetal and adult state. However, PMA-treated foetal cells exhibited a marked stimulation in fructose 2,6-bisphosphate concentration and in PFK-2 activity as well as in the content of hexose phosphates, while no response was found in the case of adult hepatocytes. Moreover, the effect of PMA on foetal hepatocytes was suppressed when cells were incubated with cycloheximide, but not when this effect was elicited by bombesin or insulin. These results, and those obtained on the phosphorylation state of PFK-2, suggest that there are different pathways that modulate fructose 2,6-bisphosphate content and, therefore, the control mechanisms of glycolysis and gluconeogenesis at this regulatory step, both in adult and foetal rat liver.

The major changes observed in the transition from the foetal to the neonatal states are mainly due to nutritional and hormonal variations. At birth, the transplacental diet, rich in carbohydrates, is replaced by the high-fat diet of the suckling period. Late in the prenatal state, a series of metabolic modifications in the liver may contribute to the development of the physiological adaptation of this organ for the newborn state. Marked changes concerning carbohydrate metabolism occur during the perinatal period which are controlled by hormones, among them a substantial decrease in hepatic glycolysis and in the insulin/glucagon ratio [1, 2] with an increased rate of gluconeogenesis [3].

Fructose 2,6-bisphosphate [Fru(2,6)P2] is known to play a major role in the control of glycolysis, through 6-phosphofructo-1-kinase, and gluconeogenesis, through fructose-1,6-bisphosphatase [4]. It has recently been reported that foetal hepatocytes contain a form of 6-phosphofructo-2-kinase (PFK-2), which shows no response to the glucagon-dependent inactivation exhibited by the adult hepatocytes [5]. However, in primary cultures of foetal hepatocytes (22-days old), the glucagon-induced inhibition of PFK-2 required at least 45 min to reach the half-maximal effect, suggesting that the adult form of this enzyme is rapidly induced after birth, probably due to the hormonal changes displayed during the perinatal transition [6]. As foetal and adult hepatocytes contain different isoenzymes of PFK-2, we investigated the possibility that agonists, acting through the stimulation of protein kinase C and calcium mobilization, affect the activity of PFK-2 in primary cultures of foetal and adult hepatocytes. Evidence is presented that phorbol-ester-induced activation of protein kinase C, increased Fru(2,6)P2 concentration and PFK-2 activity in foetal hepatocytes. However, the adult hepatocytes lacked this effect.

MATERIAL AND METHODS

Animals

Pregnant albino Wistar rats (300—350 g), fed on a standard laboratory diet (Sanders H-28) with food and water ad libitum, were maintained at a constant day/night rhythm and controlled temperature (21 °C). Gestational state was determined by standard criteria [7]. On day 22 of gestation, rats were decapitated and foetuses were obtained. The livers from the foetuses were used immediately. Male albino Wistar rats (180—200 g), fed and maintained under the same conditions as above, were also used in order to compare foetal with adult hepatocytes.

Materials

Substrates, antibiotics, hormones, coenzymes and enzymes were obtained from Boehringer (Mannheim, FRG) or Sigma (St. Louis, MO, USA). Standard analytical-grade laboratory reagents were obtained from Merck (Darmstadt, FRG). Tissue-culture dishes were from Costar (Cambridge, MA, USA). Foetal-calf serum was from Biochrom (Berlin, FRG). Media
were purchased from Amersham International (Amersham, Buckinghamshire, England).

**Isolation of hepatocytes**

Isolated hepatocytes from 22-day-old foetal livers were obtained by a non-perfusion collagenase-dispersion technique as described previously [5]. Isolation of adult hepatocytes was carried out from 3-month-old rats by the classic collagenase-perfusion method [8].

**Culture conditions and preparation of cell extracts**

Foetal and adult hepatocytes (10^6 cells) were placed in 1.5-cm-deep tissue-culture dishes previously coated with collagen [6], which contained 1 ml Dulbecco's modified Eagle's medium (DMEM) supplemented with 2% (by vol.) foetal calf serum. The dishes were incubated at 37°C for 2 h in air with 5% CO₂ to facilitate the attachment of the cells. At the end of the 2-h period the medium was aspirated together with the unattached cells, the plates washed with 1 ml DMEM, and the medium replaced by 1 ml DMEM containing 0.2% (by vol.) fatty-acid-free bovine serum albumin and incubated for 4 h. In experiments with [32P]phosphate-labelled cells, hepatocytes were cultured in 6-cm-wide dishes. After 2 h, the medium was replaced by DMEM containing 0.2% (by vol.) bovine serum albumin and 150 μCi/ml [32P]phosphate. The incubation was maintained for 2 h to equilibrate the radiolabelling of the adenine nucleotide pool with [32P]phosphate. 4β-phorbol 12-myristate 13-acetate (PMA), bombesin and insulin were added to the dishes so that the total volume of the medium was not changed by more than 2%. PMA was prepared (1 mg/ml) in dimethyl sulfoxide.

Bombesin and insulin were dissolved in sterile water in the appropriate concentration and were passed through a sterile 0.22-μm-pore filter. In the experiments using cycloheximide, cells were incubated with it for 15 min before the addition of ligands. At the indicated times the medium was aspirated and the cell layer was used for the measurement of metabolites and enzymes.

The specific radioactive peak of the [γ-32P]ATP pool was determined as follows: Samples of cell incubations were deproteinized in 200 μl 0.5 M perchloric acid and sonicated (four pulses of 30 s at 22 kHz). After centrifugation in an Eppendorf centrifuge the supernatants were neutralized with 0.5 M K₂CO₃ and processed according to Garrison [9]. ATP was purified by HPLC with a Supelcosil-LC-SAX column (Sulpco) at a flow rate of 1 ml/min, using an isocratic gradient of 0.4 M NaH₂PO₄, pH 4.5, and the absorbance at 260 nm was continuously recorded. Appropriate internal standards of ATP were used to quantify the amount of ATP in the samples. The specific activity of [γ-32P]ATP was determined after transference of the γ-phosphate of the purified ATP to glucose in the presence of hexokinase, and the measurement of the radioactivity incorporated into glucose 6-phosphate (Glc6P) [9].

To measure the concentration of Fru(2,6)P₂, the attached cells were extracted with 0.4 ml 50 mM NaOH and maintained for 10 min in a water bath at 80°C. For the measurement of enzyme activities, PFK-2 and fructose-2,6-bisphosphatase [Fru(2,6)P₆ase], the cells were washed once with the DMEM without serum or albumin and scraped off the dishes in 1 ml ice-cold buffer containing 100 mM NaF, 1 mM EDTA, 1 mM dithiothreitol and 20 mM KH₂PO₄, pH 7.4, and homogenized by sonication (four periods of 20 s) at maximal intensity. The suspensions were centrifuged at 105,000 g for 30 min and the supernatants were then fractionated after precipitation with 15% poly(ethylene glycol) [6]. After resuspension of the pellets in the extraction medium, PFK-2 activity was measured at pH 6.6 [in the presence of 5 mM Mg⁺ · ATP and 1 mM fructose 6-phosphate (Fru6P)] and at pH 8.5 (5 mM Mg⁺ · ATP and 5 mM Fru6P). Fru6P was in a 1:3 ratio with Glc6P to prevent changes in its concentration. Fru(2,6)P₆ase activity was determined in the same fraction.

To determine the phosphorylation state of PFK-2, hepatocytes were incubated in the presence of [32P]phosphate as described above, except that the poly(ethylene glycol) pellet was resuspended in 500 μl 200 mM NaCl, 1 mM dithiothreitol, 30 mM NaF, 1 mM MgCl₂, 1 mM potassium phosphate, 50 μM Fru6P and 20 mM Heps, pH 7.4 (medium A), then mixed thoroughly with 1 ml medium A containing 100 mg wet blue-Sepharose. The blue-Sepharose gel was washed 5–10 times, in batches, with 3 ml medium A containing 0.8 M NaCl, until no radioactivity emerged. Under these conditions, at least 85% of PFK-2 activity was retained by the gel. This recovery was not affected by the hormonal treatment of the hepatocytes and therefore the phosphorylation state of the enzyme had no influence on its binding to the blue-Sepharose gel. PFK-2 activity was extracted from the gel after heating at 80°C for 5 min with 250 μl Laemmli’s sample buffer followed by SDS-PAGE (10% gel) and stained with Coomassie brilliant blue [10], dried and exposed to X-ray film. Gels were loaded with amounts of protein corresponding to the poly(ethylene glycol) fractions. The protein content at this step was approximately 200 μg. Alternatively, the proteins from the gels were transferred to nitrocellulose membranes (0.5 mA/cm²; 15 h) and submitted to Western-blot analysis using a polyclonal antibody recognizing both foetal and adult PFK-2. In the latter case, the stained blots were excised and the radioactivity corresponding to the PFK-2 band was measured.

**Preparation of anti-PFK serum**

Adult liver PFK-2 was purified according to Rider et al. [11]. Antibodies were prepared by multiple intradermal injections of an emulsion of 0.5 mg purified enzyme in 0.5 ml Freund’s complete adjuvant into male New Zealand rabbits, followed by 0.2-mg booster injections of enzyme/adjuvant every two weeks. Blood was collected and the serum was separated and stored at −70°C. This antibody failed to completely precipitate PFK-2, either from foetal or adult hepatocytes, but recognized both enzymes in Western-blot analysis.

**Measurements of metabolites and enzyme activities**

Fru(2,6)P₂ was measured by the method of Van Schaftingen et al. [12]. Glc6P and Fru6P concentrations were determined enzymically as previously described [13]. Protein concentration was determined by the method of Bradford [14] with bovine serum albumin as standard. PFK-2 activity was assayed as described by Martin-Sanz et al. [6]. 1 U of PFK-2 activity is the amount of enzyme that catalyzes the formation of 1 μmol Fru(2,6)P₂/min. Fru(2,6)P₆ase activity was assayed following the method of Van Schaftingen et al. [15] in which the release of [32P]phosphate from [2-32P]Fru(2,6)P₂ was measured. [2-32P]Fru(2,6)P₂ was prepared as described by Rider et al. [11]. 1 U of Fru(2,6)P₆ase activity is the amount of
enzyme that catalyzes the release of $1 \mu$mol $[^{32}P]phosphate/\min$.

Protein-kinase-C activity was measured after homogenization of the cells in 2 ml 0.25 M sucrose, 10 mM 2-mercaptoethanol, 1 mM EGTA, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 20 $\mu$g leupeptin and 20 mM Hepes, pH 7.4. The homogenate was centrifuged at 105000 g for 30 min and the soluble and particulate fractions were purified by DEAE-cellulose (DE52) chromatography [16]. The particulate activity was extracted with homogenization buffer supplemented with 0.15% Nonidet P40. The activity of protein kinase C was followed by phosphorylation of histone H1 in the absence or presence of $Ca^{2+}$, phosphatidylserine and diacylglycerol as previously described [16].

RESULTS

Fru(2,6)$P_2$ in foetal and adult hepatocytes

To investigate whether the induced activation of inositol phospholipids turnover, or the direct activation of PKC affects the activity of PFK-2 and the concentration of Fru(2,6)$P_2$, foetal and adult hepatocytes were incubated with PMA, bombesin and insulin. Fig. 1 shows the effect of PMA, bombesin and insulin on the concentration of Fru(2,6)$P_2$ in foetal and adult hepatocytes incubated in parallel at $37^\circ C$ for up to 4 h. In foetal hepatocytes, Fru(2,6)$P_2$ showed a marked and progressive increase, due to the effect of these substances, during the first 4 h of incubation. The increased Fru(2,6)$P_2$ content was maintained for up to 24 h. However, in hepatocytes from adult rats, PMA failed to show any effect on Fru(2,6)$P_2$ content. The activation of protein kinase C after exposure of hepatocytes to PMA was assessed after determination of the subcellular distribution of this enzyme, both in adult and foetal cells, since the activating effect of PMA on protein kinase C involves the translocation of this enzyme from the cytosol to the membranes [17]. As shown in Table 1, at least 80% of the total activity was found in the membranes 2 min after exposure to phorbol esters.

The increase in the concentration of Fru(2,6)$P_2$ in cultured foetal hepatocytes in response to PMA, bombesin and insulin was time dependent and required at least 4 h to reach a maximum (Fig. 1A). The changes observed in Fru(2,6)$P_2$ concentration after exposure of foetal or adult hepatocytes to these ligands (bombesin, insulin and PMA in the case of foetal hepatocytes), followed nearly identical time courses. Moreover, the time required to obtain the half-maximal expression of this effect was also similar (1.5 h). In contrast, adult hepatocytes incubated in the presence of PMA failed to show changes in their content of Fru(2,6)$P_2$ but surprisingly, as in the foetal state, bombesin retained its ability to promote a rise in the level of this metabolite (Fig. 1B). This is despite the fact that both PMA and bombesin promote the activation of protein kinase C either directly, as in the case of PMA [17, 18] (Table 1) or indirectly through the metabolites derived from inositol phospholipids turnover (bombesin) [19]. Insulin produced the already described increase in Fru(2,6)$P_2$ [20, 21].

Table 2 shows the concentrations of Fru(2,6)$P_2$ in foetal and adult hepatocytes incubated for 4 h under the different experimental conditions, as well as when bombesin or insulin were incubated with PMA. No apparent synergism between these ligands was observed in cells from either foetal or adult rats.

To test whether the effects of PMA, bombesin and insulin depend on protein synthesis, primary cultures of foetal and adult hepatocytes were incubated for 15 min with 1 $\mu$M cycloheximide before the addition, at saturating concentrations, of the ligands (160 nM PMA: 100 nM bombesin; 100 nM insulin). The incubation was followed for 4 h and samples were collected to determine Fru(2,6)$P_2$ concentration (Table 2) and PFK-2 activity (Table 3). Cycloheximide slightly decreased the Fru(2,6)$P_2$ content in control cells (from 100% to 91%) and suppressed the increase in Fru(2,6)$P_2$ concentration elicited by PMA in foetal hepatocytes. In contrast to this effect, exposure of both foetal and adult hepatocytes, previously treated with cycloheximide, to bombesin or insulin did not show any influence on the intracellular concentration of Fru(2,6)$P_2$. The effects of cycloheximide on Fru(2,6)$P_2$ were parallel to changes in PFK-2 activity.

Hexose phosphates

The concentration of Fru(2,6)$P_2$ may be modulated by the activity of PFK-2/Fru(2,6)$P_2$ase and by the changes in the
Table 2. Hexose phosphate and Fru(2,6)P₂ content in foetal and adult hepatocytes incubated in the presence of PMA, bombesin and insulin and the effects of cycloheximide. Primary cultures of hepatocytes from foetal and adult rats were incubated in the medium (DMEM supplemented with 0.2% fatty-acid-free bovine serum albumin) for 2 h. The medium was replaced and cells were incubated in the presence of the indicated substances for 4 h. Incubation with 1 μM cycloheximide was carried out for 15 min before the addition of PMA, insulin and bombesin. Results are means ± SEM of four experiments. n.d., not determined.

<table>
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<tr>
<th>Hepatocyte type</th>
<th>Addition(s)</th>
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<th>Fru(2,6)P₂ content</th>
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<td></td>
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<td>pmol/mg protein</td>
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<td>Foetal</td>
<td>none</td>
<td>48 ± 1</td>
<td>11 ± 1</td>
</tr>
<tr>
<td></td>
<td>160 nM PMA</td>
<td>79 ± 2 *</td>
<td>39 ± 3 *</td>
</tr>
<tr>
<td></td>
<td>100 nM bombesin</td>
<td>70 ± 6 *</td>
<td>36 ± 3 *</td>
</tr>
<tr>
<td></td>
<td>100 nM bombesin/160 nM PMA</td>
<td>n.d.</td>
<td>36 ± 3 *</td>
</tr>
<tr>
<td></td>
<td>100 nM insulin</td>
<td>62 ± 7</td>
<td>34 ± 3 *</td>
</tr>
<tr>
<td></td>
<td>100 nM insulin/160 nM PMA</td>
<td>n.d.</td>
<td>31 ± 2 *</td>
</tr>
<tr>
<td>Adult</td>
<td>none</td>
<td>79 ± 5</td>
<td>18 ± 2</td>
</tr>
<tr>
<td></td>
<td>160 nM PMA</td>
<td>62 ± 5 *</td>
<td>20 ± 2</td>
</tr>
<tr>
<td></td>
<td>100 nM bombesin</td>
<td>161 ± 18 *</td>
<td>40 ± 5 *</td>
</tr>
<tr>
<td></td>
<td>100 nM bombesin/160 nM PMA</td>
<td>n.d.</td>
<td>42 ± 4 *</td>
</tr>
<tr>
<td></td>
<td>100 nM insulin</td>
<td>128 ± 8 *</td>
<td>34 ± 4 *</td>
</tr>
<tr>
<td></td>
<td>100 nM insulin/160 nM PMA</td>
<td>n.d.</td>
<td>40 ± 5 *</td>
</tr>
</tbody>
</table>

* Significant difference from control (none), *P* < 0.001.

* Significant difference from control (none), *P* < 0.01.

* Significant difference from control (none), *P* < 0.05.

Table 3. PFK-2 and Fru(2,6)P₂ in foetal and adult hepatocytes incubated in the presence of PMA, bombesin and insulin with effects of cycloheximide. Primary cultures of hepatocytes from foetal and adult rats were incubated in the medium (DMEM supplemented with 0.2% fatty-acid-free bovine serum albumin) for 2 h. The medium was replaced and cells were incubated in the presence of the indicated substances for 4 h. Incubation with 1 μM cycloheximide was carried out for 15 min before the addition of PMA, bombesin or insulin. PFK-2 total activity was measured at pH 8.5. Results are means ± SEM of four experiments. n.d., not determined.

<table>
<thead>
<tr>
<th>Hepatocyte type</th>
<th>Addition(s)</th>
<th>PFK-2 activity</th>
<th>Fru(2,6)P₂ase activity</th>
</tr>
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<tr>
<td></td>
<td></td>
<td>control</td>
<td>cycloheximide</td>
</tr>
<tr>
<td></td>
<td></td>
<td>mU/g protein</td>
<td></td>
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<tr>
<td>Foetal</td>
<td>none</td>
<td>24 ± 3</td>
<td>22 ± 3</td>
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<tr>
<td></td>
<td>160 nM PMA</td>
<td>41 ± 3 *</td>
<td>16 ± 2 *</td>
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<tr>
<td></td>
<td>100 nM bombesin</td>
<td>37 ± 2 *</td>
<td>32 ± 4</td>
</tr>
<tr>
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<td>100 nM bombesin/160 nM PMA</td>
<td>45 ± 3 *</td>
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<tr>
<td></td>
<td>100 nM insulin</td>
<td>29 ± 3</td>
<td>28 ± 3</td>
</tr>
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<td>100 nM insulin/160 nM PMA</td>
<td>37 ± 4 *</td>
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<td>Adult</td>
<td>none</td>
<td>38 ± 4</td>
<td>35 ± 4</td>
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<td>160 nM PMA</td>
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<td>100 nM bombesin</td>
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<td>100 nM insulin/160 nM PMA</td>
<td>56 ± 6 *</td>
<td>n.d.</td>
</tr>
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</table>

* Significant difference from control (none), *P* < 0.001.

* Significant difference from control (none), *P* < 0.01.

* Significant difference from control (none), *P* < 0.05.

The differing behaviours of foetal and adult hepatocytes with PMA agrees with the previously described results.

**PFK-2 and Fru(2,6)P₂ase activities**

The maximal activity of PFK-2 was measured in primary cultures of foetal and adult hepatocytes incubated for 4 h in the presence of PMA, bombesin and insulin. This sampling time was chosen because of the important changes that occur during this period [6]. As shown in Table 3, an increase in the concentration of Fru6P. For this reason, hexose-phosphate (Glc6P and Fru6P) concentration was also determined. The results obtained are shown in Table 2. Hexose-phosphate content increases in foetal hepatocytes incubated in the presence of PMA (165%, *P* < 0.01), bombesin (146%, *P* < 0.05) and insulin (129%), which provides a mechanism to explain the effect of insulin on the Fru(2,6)P₂ concentration. However, in adult hepatocytes, the hexose-phosphate concentration decreases in response to PMA (78%) and increases in the presence of bombesin (204%, *P* < 0.001) and insulin (162%, *P* < 0.01).
activity of PFK-2 was evident in foetal hepatocytes when incubated in the presence of PMA (171%, P < 0.01) and bombesin (154%, P < 0.01). The increase observed by the effect of insulin (121%) was less evident. For PMA and bombesin these results are in agreement with the changes in the concentration of Fru(2,6)P2. The same holds true for adult hepatocytes in which PMA did not produce changes in the concentration of Fru(2,6)P2 or in the activity of PFK-2.

The Fru(2,6)Pase activity was determined in foetal and in adult hepatocytes and the results obtained showed that in the foetal cells the effect of bombesin produced a significant increase while no changes were seen with PMA. However, this enzyme activity was less than the control when adult hepatocytes were incubated in the presence of PMA or bombesin (Table 3). This suggests the occurrence of a Fru(2,6)Pase activity in foetal hepatocytes, different from the PFK-2/Fru(2,6)Pase bifunctional enzyme.

The activity ratio PFK-2/Fru(2,6)Pase was 70-fold higher in foetal than in adult hepatocytes (Table 3), due to the low Fru(2,6)Pase activity in foetal cells. Exposure of foetal hepatocytes to PMA produced an increase (152%) in this ratio through a rise in the PFK-2 activity, whereas in adult cells the increase in this ratio was mediated through a decreased Fru(2,6)Pase activity. These data agree with previous results concerning the low activity of Fru(2,6)Pase in foetal liver [5] and in hepatoma cells [22].

**In vivo phosphorylation of PFK-2**

To determine the phosphorylation state of PFK-2, primary cultures of foetal and adult hepatocytes were radiolabelled with [32P]phosphate, and the specific activity of [γ-32P]ATP was determined at sampling times (10 min and 4 h after ligand addition), as described in Materials and Methods. PMA, bombesin and insulin, as well as glucagon (as a positive control of adult PFK-2 phosphorylation), were added to [32P]phosphate-labelled cells, prepared as described above. Samples were collected to determine the extent of PFK-2 phosphorylation and the specific activity of ATP. The cell extracts were fractionated by poly(ethylene glycol) and absorbed onto blue-Sepharose to decrease the background of phosphoproteins. By using this procedure only, 5—15% of PFK-2 activity was detected in the supernatants. In foetal hepatocytes, 10 min of incubation with glucagon produced a marked increase in the phosphorylation state of a 54-kDa protein and a lesser phosphorylation of two proteins of 60 kDa and 52 kDa (Fig. 2). When foetal hepatocytes were stimulated with PMA or bombesin, the same proteins were phosphorylated, although the incorporated phosphate was less than after incubation with glucagon. Insulin failed to show changes in the phosphorylation of proteins purified with blue-Sepharose. When the same experiment was carried out with adult hepatocytes, only glucagon produced a clear phosphorylation of a protein of 54 kDa [5, 6]. The specific activity of [γ-32P]ATP was 0.36 ± 0.03 μCi/nmol ATP and 0.15 ± 0.03 μCi/nmol ATP for foetal and adult hepatocytes, respectively. The corresponding values for each condition are given in the legend to Fig. 2.

To identify the band corresponding to PFK-2 in foetal hepatocytes, samples were processed by SDS/PAGE followed by Western-blot analysis using a polyclonal rabbit anti-(rat liver PFK) serum that recognized both the foetal and adult enzymes. Only the band corresponding to 54 kDa was stained. This is an additional proof that the radioactivity measured for the 54-kDa protein, effectively corresponds to PFK-2. In samples obtained after 4 h of incubation with the hormones, the protein bands recognized by Western-blot analysis were excised and the radioactivity associated with the stained bands (Table 4) shown to exhibit a pattern similar to those given in Fig. 2. The specific activity of [γ-32P]ATP for each incubation is shown in Table 4.

PFK-2 activity, expressed as activity at pH 8.5/activity at pH 6.6, was determined in the poly(ethylene glycol) extracts, since in adult liver, the activity at pH 8.5 represents the total activity of PFK-2 independently of the phosphorylation state of the enzyme, and at pH 6.6, the activity of PFK-2 decreases when the enzyme is phosphorylated [23]. In foetal hepatocytes, no significant changes were observed in this activity ratio of PFK-2 regardless of the phosphorylation state of the enzyme. In adult hepatocytes only glucagon increased the activity ratio (Table 4). Since phosphorylation of PFK-2 from foetal hepatocytes is observed after 10 min of exposure to PMA or bombesin, it is concluded that phosphorylation itself is not involved either in the changes in Fru(2,6)P2 concentration (Fig. 1) or in the kinetic variations observed in PFK-2 (Table 3).

Hepatocytes from foetal and adult rats were incubated for 4 h in the presence of increasing concentrations of PMA, bombesin and insulin and the content of Fru(2,6)P2 was determined (Fig. 3). Fru(2,6)P2 production was similar in both foetal and adult hepatocytes exposed to bombesin (Fig. 3B) or insulin (Fig. 3C). However, the PMA-induced increase in Fru(2,6)P2, which reached a maximum in foetal hepatocytes at 40 nM PMA, remained unchanged when adult cells were incubated with increased concentrations of this ligand (Fig. 3A).

**DISCUSSION**

We studied the effect of PMA, bombesin and insulin on the PFK-2/Fru(2,6)Pase system in primary cultures of foetal and adult hepatocytes for up to 8 h. The biological responses elicited by these substances, involving changes in the PFK-2/Fru(2,6)Pase activity and Fru(2,6)P2 concentration, required at least 4 h to be fully expressed and, therefore, the use of these primary cultures provides an efficient model for study.

The present results suggest that growth factors and tumour promoters modulate Fru(2,6)P2 concentration in two ways; either by an increase in PFK-2 activity or by changes in the
Table 4. Phosphorylation of PFK-2 in hepatocytes incubated with PMA, insulin, bombesin or glucagon. Hepatocytes (5 x 10^7 cells) were radiolabelled with [32P]phosphate for 2 h prior to the addition of the ligands. After 4 h, cells were homogenized and the cell extracts were fractionated by poly(ethylene glycol) and affinity chromatography on blue-Sepharose. The SDS/PAGE was followed by Western-blot analysis using anti-PFK-2 serum, and the stained bands were excised and the radioactivity detected. Radioactivities are means ± SEM of three different experiments. The specific radioactivities of [γ-32P]ATP are the means of two independent determinations and the ratio of PFK-2 activity measured at pH 8.5 and pH 6.6 was carried out as described in Materials and Methods.

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<td></td>
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<tr>
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</tr>
<tr>
<td>100 nM glucagon</td>
<td>3122 ± 410*</td>
<td>1.0</td>
</tr>
<tr>
<td>100 nM bombesin</td>
<td>873 ± 74*</td>
<td>1.0</td>
</tr>
<tr>
<td>100 nM insulin</td>
<td>159 ± 26</td>
<td>1.1</td>
</tr>
</tbody>
</table>

* Significantly different to control, P < 0.001.

Fig. 3. Dose/response curves of PMA (A), bombesin (B) and insulin (C) on Fru(2,6)P_2 concentrations in foetal (○) and adult (▲) hepatocytes. Cells were incubated in the medium (DMEM supplemented with 0.2% fatty-acid-free bovine serum albumin) for 2 h. The medium was replaced and cells were incubated for 4 h in the presence of several concentrations of agonists. Results are means ± SD of four replicates in a representative experiment. * P < 0.001.

The mechanism by which protein-kinase-C activation increases PFK-2 activity is still not clear, although evidence has been provided showing a direct effect of protein kinase C on the phosphorylation state [27, 28] that, according to Kitamura et al. produced a twofold increase in V_max without affecting the affinity for Fru6P in PFK-2 from bovine heart [28]. In our studies, incubation of foetal hepatocytes with PMA and bombesin produced a clear in vivo phosphorylation of PFK-2, whereas this response was absent in adult hepatocytes. Glucagon, used as a control for the phosphorylation of the adult enzyme, also promoted the phosphorylation of PFK-2 in foetal cells, suggesting that this enzyme is a substrate for both protein kinase C and cyclic-AMP-dependent protein kinase as described for the bovine heart enzyme [28]. However, PFK-2 from foetal hepatocytes, incubated with either PMA or glucagon did not exhibit changes in the activity ratio (pH 8.5/pH 6.6; Table 4), nor in their affinity for Fru6P nor in their sensitivity to sn-glycerol 3-phosphate inhibition (data not shown).

The bombesin-transduction pathway involves the activation of a inositol-phospholipid-specific phospholipase C, producing an increase in the diacylglycerol content in the plasma membrane and a rise in the free cytosolic calcium concentration [19]. These second messengers are activators of protein kinase C [17]. However, the involvement of protein kinase C in the bombesin-induced rise in the level of concentration of Fru6P, and these mechanisms may influence the glycolytic and gluconeogenic fluxes at the level of Fructose-6-phosphate (Fructose-6-P) conversion [24]. The most notable differences between the foetal and adult cells in the PFK-2/Fru(2,6)P_2ase system were in the response following the activation of protein kinase C by phorbol esters. Adult hepatocytes lacked this response. However, foetal liver cells exhibited a marked enhancement in Fru(2,6)P_2, in hexose-phosphate content and in PFK-2 activity when exposed to PMA at concentrations in the range of maximal protein-kinase-C activation (160 nM). As protein kinase C is the biological receptor of phorbol esters, it is suggested that the effects of PMA are solely mediated by the activation of protein kinase C [17]. However, the involvement of protein kinase C in the bombesin-induced rise in the level of PFK-2 activity is still not clear, although evidence has been provided showing a direct effect of protein kinase C on the phosphorylation state [27, 28] that, according to Kitamura et al. produced a twofold increase in V_max without affecting the affinity for Fru6P in PFK-2 from bovine heart [28]. In our studies, incubation of foetal hepatocytes with PMA and bombesin produced a clear in vivo phosphorylation of PFK-2, whereas this response was absent in adult hepatocytes. Glucagon, used as a control for the phosphorylation of the adult enzyme, also promoted the phosphorylation of PFK-2 in foetal cells, suggesting that this enzyme is a substrate for both protein kinase C and cyclic-AMP-dependent protein kinase as described for the bovine heart enzyme [28]. However, PFK-2 from foetal hepatocytes, incubated with either PMA or glucagon did not exhibit changes in the activity ratio (pH 8.5/pH 6.6; Table 4), nor in their affinity for Fru6P nor in their sensitivity to sn-glycerol 3-phosphate inhibition (data not shown).

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Fructose-2,6-bisphosphate (Fru(2,6)P₂) in adult hepatocytes seems unlikely, since direct activation of protein kinase C by PMA produced a lower response than that observed with bombesin and both failed to promote the phosphorylation of the enzyme. To explain the effect of bombesin in these cells, the Ca²⁺ mobilization elicited by this agonist should be considered because bombesin, acting like other Ca²⁺-mobilizing hormones, may stimulate glycogen degradation and therefore produce a sustained increase in the hexose-phosphate level that may explain its effects on the concentration of Fru(2,6)P₂. Another possible explanation for the bombesin-induced enhancement of PFK-2 activity is that the second messengers elicited by this agonist might be different from those obtained with bombesin. This is in agreement with a previous study on adult hepatocytes cultured for 48 h [20], although in that report, insulin only increased PFK-2 activity without affecting the concentration of Fru6P. However, PFK-2 was not phosphorylated in hepatocytes incubated with insulin. The rise in the level of hexose phosphate observed in our experiments may be explained by the use of shorter incubation times with hepatocytes which would contribute to the maintenance of the hepatic phenotype. Concerning the mechanism by which insulin increases PFK-2 activity, it is suggested that the hormone helps to stabilize the enzyme, since in diabetic animals the decreased PFK-2 activity is largely due to a decrease in the enzyme content but not in the mRNA for PFK-2/Fru(2,6)P₂ase [29].

In contrast to the adult hepatocytes, in foetal cells the effect of insulin seems to be exclusively mediated by the increase in the concentration of hexose phosphate. However, in a previous work with foetal hepatocytes [6], no changes were observed in Fru(2,6)P₂ concentration in response to insulin, probably due to the use of different experimental conditions (DMEM instead of Leibowitz L-15 media and recombinant human insulin instead of the porcine hormone). Moreover, the results shown in Fig. 3 suggest that the effect of insulin may be due, in part, to binding to insulin-like-growth-factor receptors, in view of the concentration of insulin (100 nM) used in this study. The different regulation of PFK-2 in foetal or adult hepatocytes in response to insulin and glucagon [6], as well as the differential phosphorylation pattern observed in vivo after activation of the cyclic-AMP-dependent protein kinase and protein kinase C, emphasises our proposal that there is a foetal form of PFK-2 with regulatory properties different from those found in the adult liver enzyme [5, 6].

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REFERENCES