Pituitary adenylate cyclase-activating polypeptide induces expression of corticosteroid-binding globulin in cultured fetal hepatocytes: synergy with tri-iodothyronine

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The purpose of the present study was to determine whether functional receptors for pituitary adenylate cyclase-activating polypeptide (PACAP) are expressed in cultured rat fetal hepatocytes and eventually play a role in regulating gene expression of corticosteroid-binding globulin (CBG). We found PACAP38 and PACAP27 to elevate cAMP levels in hepatocytes in a dose-dependent manner, with a plateau being achieved at 10 nM and EC_{50} values of about 0.5–1 nM. PACAP failed to alter the turnover of inositol phosphates, whereas PACAP and VIP stimulated cAMP accumulation in an equipotent manner, suggesting the presence in these cells of type II receptor isoforms. As revealed by measurements of both CBG mRNA levels and concentrations of binding sites, long-term treatment of fetal cells

with 10 nM PACAP, although resulting in partial desensitization of peptide-induced cAMP accumulation, caused a significant 3-fold elevation in CBG synthesis. This stimulatory influence of PACAP was mimicked by the cell permeant $N^6,2'$ -*O*-dibutyryl-adenosine 3',5'-phosphate (dbcAMP). Treatment of hepatocytes with tri-iodothyronine (T_3) enhanced CBG expression and, most interestingly, appeared to synergize with PACAP to elicit a 2–3-fold amplification of CBG synthesis. This study thus provides first evidence for the up-regulation by PACAP and cAMP of CBG expression in fetal hepatocytes and for T_3 's playing a synergistic role in enhancing PACAP-induced synthesis of the binder.

INTRODUCTION

Pituitary adenylate cyclase-activating polypeptides (PACAPs) are new members of the vasoactive intestinal polypeptide (VIP)/glucagon/secretin family of peptides (reviewed in [1,2]). They are present in tissues as two forms with 38 and 27 amino acid residues (PACAP38 and PACAP27), which are distributed in several areas of the central nervous system, as well as outside the brain in tissues such as the pituitary, adrenal gland, testis, pancreas and gastrointestinal system.

The effects of PACAPs are mediated through at least two types of receptor, which are distinguished by their affinity for VIP. The type I receptors prefer PACAPs over VIP, whereas type II receptors bind PACAPs and VIP with similar affinities (reviewed in [3]). Both forms of receptor stimulate adenylate cyclase activity, but type I and some new isoforms of type II in addition couple positively to phospholipase C (PLC) [4,5]. These receptors are differentially distributed in tissues: those of class I are present in the brain, pituitary and testis, whereas class II types are expressed in the lung and the gastrointestinal tract [1]. In the liver, receptors specific for PACAP and receptors sharing binding with VIP have been demonstrated [5–8].

For the past several years we have been engaged in a project aimed at characterizing the various regulatory factors that may be directly involved in the control of corticosteroid-binding globulin (CBG) in cultured hepatocytes [9–11]. CBG is a non-receptor binder for glucocorticoids and progesterone, which is synthesized mainly in the liver. It is present not only in the plasma, but also inside cells as a result of either internalization or intracellular synthesis (reviewed in [12]). Moreover, the protein has the potential to interact with specific cell membrane receptors and eventually alter cAMP formation [13,14]. Because of the presence of PACAP receptors in the liver and evidence that

cAMP actually plays a critical role in gene expression in hepatocytes [15–17], we launched the present study to investigate the effect of PACAP on the expression of CBG in cultured rat fetal hepatocytes and the possible involvement of cAMP in this process. Furthermore, having previously demonstrated a direct stimulatory effect of tri-iodothyronine (T_3) on the biosynthesis of CBG [10], we also tested hepatocytes for a possible interplay between PACAP and T_3 .

MATERIALS AND METHODS

Animals and reagents

Pregnant Wistar female rats, weighing 230–250 g, were used throughout. The time of conception of these rats (day 0) was assessed from sperm-positive vaginal smears on the day after the mating night. PC12 cells were obtained from the European Collection of Animal Cell Cultures (Salisbury, Wilts., U.K.).

Williams' medium E, Dulbecco's modified Eagle's medium/Ham's mixture F-12, DNase I, insulin, T_3 and $N^6,2'$ -*O*-dibutyryl-adenosine 3',5'-phosphate (dbcAMP) were purchased from Sigma Chimie (LaVerpilleries, France), and [$1,2$ - 3H](N)-corticosterone (50.5 Ci/mmol) and [α - ^{32}P]dCTP (3000 Ci/mmol) from New England Nuclear (Du Pont de Nemours). *myo*-[3H]Inositol (102 Ci/mmol) and Amprep (RPN 1908) minicolumns were obtained from Amersham France SA. PACAP38, PACAP27 and VIP were from Bachem (Bale Biochimie). Rolipram, a selective inhibitor of type IV cAMP-phosphodiesterase, was a gift from Schering AG (Berlin, Germany).

Cultures of fetal hepatocytes and PC12 cells

Livers of fetuses from 16- or 17-day pregnant rats were dissected out under sterile conditions and processed for cell

Abbreviations used: CBG, corticosteroid-binding globulin; CRE, cAMP response element; dbcAMP, $N^6,2'$ -*O*-dibutyryl-adenosine 3',5'-phosphate; PACAP, pituitary adenylate cyclase-activating polypeptide; PLC, phospholipase C; T_3 , tri-iodothyronine; VIP, vasoactive intestinal polypeptide.

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culture as already described [10]. Briefly, tissues were dissociated with collagenase (50 mg per 100 ml) in the presence of DNase I (1 mg per 100 ml) in HEPES buffer at pH 7.3. Dissociated cells (corresponding to 1.0–1.5 liver equivalents) were cultured in 6-well or 12-well culture plates (Primaria, Falcon) and in Williams' medium E supplemented with 5% (v/v) fetal calf serum, 1 μ M insulin, 0.3 mM 2-mercaptoethanol and antibiotics for 2 days. After that time period the culture medium was changed for serum-free medium (containing 0.1% BSA) and the cells were incubated for a further 2 days. The various substances to be tested were included in the culture media from the first day and throughout, with media being changed daily. That period of treatment was required to register clear-cut effects on CBG biosynthesis by hepatocytes in culture [10] and actually reflects the time lag likewise seen *in vivo* in rats administered with corticosterone and thyroxine or after adrenalectomy [18]. Cellular DNA contents were measured by the bis-benzimide technique [19] and taken as an index of cell number.

Phaeochromocytoma cells PC12 were cultured in Dulbecco's modified Eagle's medium/Ham's mixture F-12, supplemented with 10% (v/v) fetal calf serum and antibiotics.

Measurements of cAMP formation

Hepatocytes were washed and incubated in serum-free Williams' medium E, containing 0.1% BSA and 25 μ M Rolipram, in the absence and presence of graded doses of PACAPs. After the indicated times, the reaction was arrested by addition of 1 volume of ice-cold 0.2 M HCl. Cells were further disrupted by sonication and the suspensions were centrifuged at 10000 *g* for 10 min. The resulting supernatants were stored at -25°C for measurement of cAMP by radioimmunoassay [20].

Measurement of inositol phosphate accumulation

After 1 day in culture, hepatocytes were cultured for a further 2 days in the presence of *myo*-[^3H]inositol (4 $\mu\text{Ci}/\text{ml}$) in *myo*-inositol-free culture medium. Parallel experiments were conducted with PC12 chromaffin cells, which were taken here as a model for PACAP-induced phosphoinositide breakdown [21]. The cells were then washed and treated with 10 mM LiCl for 10 min before being challenged with 10 nM PACAP38 for 1 min. The cells were homogenized in ice-cold 5% (v/v) perchloric acid and the supernatants, obtained after centrifugation at 1200 *g* for 5 min, were neutralized with 10 M KOH. The extracts were finally filtered through Amprep minicolumns and inositol phosphate species eluted by means of a stepwise gradient of ammonium formate/formic acid, as reported [22].

Northern blots and measurement of CBG mRNA

Total RNA was extracted from cultured hepatocytes as already described [10,11]. For Northern blot analysis, a radioactive CBG probe labelled with [α - ^{32}P]dCTP was used to detect the presence of mRNA. Hybridization was performed at a final medium stringency of $0.1 \times (0.15 \text{ M NaCl}/0.015 \text{ M sodium citrate})$, 0.1% SDS at 50°C for 30 min, and measurement of mRNA was performed as previously reported. The actual concentrations of material applied to the membranes were measured and these values used to correct CBG mRNA data, which were expressed in arbitrary units for a constant concentration of total RNA (15 μg).

Measurements of CBG binding

Aliquots of 0.2 ml of incubation medium from 1-day-cultured cells were labelled with 1.5 pmol of [^3H]corticosterone, in the

absence or presence of increasing concentrations of unlabelled corticosterone. Parallel samples contained a 1000-fold excess of unlabelled steroids to permit correction for non-specific binding. Assay tubes were first held at 30°C for 20 min and then maintained in an ice-cold water bath for 2 h. Bound and unbound fractions were separated by adding 50 μl of ice-cold dextran-coated charcoal mixture [0.5% dextran T70 and 5% (w/v) charcoal in 20 mM Tris/HCl buffer, pH 7.2] to the assay tubes. After being vortexed and maintained in an ice-bath for 10 min, the tubes were centrifuged at 1500 *g* for 10 min and 150 μl aliquots of the supernatants were taken out and counted for bound radioactivity, with a counting efficiency of 32–35%.

Statistics

Statistical significance of data was assessed by means of analysis of variance, followed by the Student–Newman–Keuls test for multiple comparisons, using Graphpad's Instat2 software. The half-maximal values (EC_{50}) for dose–response curves, as well as the binding parameters for CBG, were calculated by non-linear regression analysis with the aid of Graphpad's Prism program. Data shown in figures are means $\pm$ S.E.M. for three determinations done in duplicate and are representative of two to four identical experiments, which yielded similar results.

RESULTS

Effect of PACAP on accumulation of cAMP and inositol phosphates in cultured hepatocytes

The time course experiment depicted in Figure 1 clearly shows that PACAP38 significantly elevated cAMP production in 1-day-

Figure 1 Time course of the effect of PACAP38 on cAMP accumulation in hepatocytes

Cells were cultured for 1 day before being challenged, or not, with 10 nM peptide in the presence of 50 μM Rolipram. Each point is the mean $\pm$ S.E.M. for four determinations done in duplicate.

Figure 2 Effect of PACAP38 on formation of inositol phosphates and stimulation by PACAP and VIP of cAMP production in fetal hepatocytes

(A) Radioactive profiles eluted from Amprep columns loaded with extracts of hepatocytes ($\square$), or PC12 cells ($\blacktriangle$), prelabelled with myo - 3H inositol. Cells were incubated for 1 min in the absence (open symbols) and presence (filled symbols) of 10 nM PACAP38. Eluted radioactive peaks were, from left to right: GroPIns, Ins(1,4) P_2 , Ins(1,4,5) P_3 , Ins(1,3,4,5) P_4 and Ins(1,3,4,5,6) P_5 (no detectable Ins3 P_1 peak was found). Basal values for PC12 cells are close to those for hepatocytes and are not shown for the sake of clarity. (B) Cells were maintained for 1 day in culture and exposed to graded doses of PACAP38 and VIP for 3 h and in the presence of 50 μ M Rolipram. Points are means $\pm$ S.E.M. for four determinations done in duplicate.

old cultured hepatocytes from 15 min onwards to at least 12 hours.

In an attempt to characterize the type of receptor present on fetal hepatocytes, we tested the effects of both VIP and PACAP on adenylate cyclase activity and of PACAP on the turnover of inositol phosphates. PACAP38 and VIP stimulated cAMP production in a dose-dependent manner, with EC_{50} values of 0.4 and 0.2 nM respectively (Figure 2B). This suggested the involvement of type II receptor isoforms, most probably not coupled to activation of PLC; an assumption that seemed to be supported by measurements of phosphoinositides' turnover (Figure 2A). Although PACAP38 was able to enhance Ins(1,4) P_2 and Ins(1,4,5) P_3 accumulation in pheochromocytoma PC12 cells (taken here as a reference model), it failed to do so in fetal hepatocytes (Figure 2A).

Dose-dependent effects of PACAP on cAMP production in hepatocytes maintained in culture for various periods

To examine whether PACAP receptors retain their functional activity during long-term culture, hepatocytes were maintained in the absence of PACAP for 3 days and the cells' responsiveness to graded doses of peptides was tested relative to 1-day-old cultures. As shown in Figure 3, both PACAP38 and PACAP27 elicited dose-dependent elevations of cAMP production in cells cultured for 1 day, which increased from a basal level of 3.2 ± 0.5 to 10.3 ± 0.6 and 12.2 ± 0.8 pmol per well respectively. Plateau values were reached with 10 nM concentrations of both peptides,

Figure 3 Dose-dependent effect of PACAP38 and PACAP27 on cAMP accumulation in hepatocytes

Cells were maintained in culture for 1 day (t_1) or 3 days (t_3) and then incubated in the presence of graded doses of PACAP38 and PACAP27, along with 50 μ M Rolipram, for 3 h. Points are means $\pm$ S.E.M. for four measurements done in duplicate.

Table 1 Effects of PACAP38 and T_3 on cAMP formation in cultured hepatocytes

Hormone-pretreated hepatocytes refer to cells exposed for 3 days to vehicle (controls), 100 nM T_3 , 10 nM PACAP38 or both hormones in combination. Both naive and pretreated cells were then challenged with the same agents, in the presence of 50 μ M Rolipram, for one additional day. Data represent the concentrations of cAMP (expressed in pmol per well) in culture media; each value is the mean $\pm$ S.E.M. for four determinations done in duplicate.

Treatment	Naive cells	Pretreated cells
None	9.9 ± 0.8	8.8 ± 0.7
T_3	11.6 ± 0.4	10.0 ± 1.1
PACAP	$28.7 \pm 1.0^*$	$18.7 \pm 0.7^{*\dagger}$
PACAP + T_3	$30.0 \pm 1.3^{*\dagger}$	$20.1 \pm 1.2^{*\dagger}$

* $P < 0.001$ compared with respective controls.

$\dagger$ $P < 0.001$ compared with related values in naive cells.

with respective EC_{50} values of 0.8 ± 0.2 and 1.8 ± 0.3 nM. Functional receptors were still expressed after 3 days in culture, as PACAP38 was able to enhance cAMP formation, with a closely similar EC_{50} value of 1.4 ± 0.2 nM compared with 1 day cultures.

Finally we show that on long-term exposure of hepatocytes to PACAP for 3 days, cells were only partly desensitized with respect to peptide-stimulated cAMP accumulation (Table 1). The cells were still capable of responding to PACAP by producing cAMP ($P < 0.001$ compared with control), although to a significantly lower level than that of their naive counterparts ($P < 0.01$).

Figure 4 Saturation binding curves of labelled corticosterone to CBG in culture media

Control, PACAP-treated and dbcAMP-treated hepatocytes were used. Cells were exposed to either 10 nM PACAP or 0.1 mM dbcAMP for 4 days. The media were changed daily and the media of the last change (between day 3 and day 4) were collected for CBG measurements. Points are means of triplicate assays of pooled media from five culture wells, and represent specific binding (total minus non-specific). Plateau values were calculated to be: 269 ± 10 , 825 ± 28 and 712 ± 39 fmol per well respectively.

Induction by PACAP of CBG expression

CBG synthesis by hepatocytes was monitored by determining both the peptide's binding capacity in incubation media and the cellular concentration of CBG mRNA. Preliminary time course experiments, in which cells were exposed to 10 nM PACAP for 1, 2, 3 and 5 days, revealed changes in $[^3\text{H}]$ corticosterone binding capacity after 3 and 5 days of treatment, with a significant effect being registered only after 5 days ($P < 0.001$ compared with controls). Values were respectively 5101 ± 610 , 3570 ± 253 , 1530 ± 202 and 550 ± 69 c.p.m. per well for control hepatocytes, and 4980 ± 313 , 3880 ± 299 , 2105 ± 160 and 3125 ± 276 c.p.m. per well for PACAP-treated hepatocytes.

Analysis by non-linear regression of $[^3\text{H}]$ corticosterone binding data clearly revealed that long-term treatment of hepatocytes with PACAP38 caused an elevation of maximum binding capacity (B_{max}) from a control level of 269 ± 9.9 to 712 ± 39 fmol per well; there was, however, no significant change in the values of the dissociation constants, K_d : 1.4 ± 0.2 and 1.8 ± 0.1 nM (Figure 4). The increase in binding capacity could not be ascribed to an increase in cell number because DNA contents were closely similar in both cases: 23.5 ± 0.5 and 25.4 ± 1.1 μg per well for control and PACAP-treated cells respectively.

In parallel with augmenting the concentration of CBG binding sites in culture medium, PACAP also induced an approx. 8-fold elevation in cellular levels of CBG mRNA, which increased from 27.7 ± 4.2 to 239.3 ± 24 arbitrary units in control and PACAP-treated hepatocytes respectively (Figure 5 and Table 2).

Figure 5 Synergistic effect of T_3 and PACAP on CBG binding capacity and mRNA levels

Hepatocytes were exposed to vehicle (column A) or 10 nM PACAP38 (column B), PACAP27 (column C) or 100 nM T_3 (D), or to PACAP38 or PACAP27 in combination with T_3 (columns E and F respectively) for up to 4 days, as described in the legend to Figure 4. Bars are means $\pm$ S.E.M. for six culture dishes measured in triplicate. Significance values: * $P < 0.05$ and *** $P < 0.001$ compared with group A; !!! $P < 0.001$ compared with groups B and C. At the top is a representative autoradiograph of a Northern blot of RNA extracts from hepatocytes treated with vehicle (I), PACAP38 (II), PACAP38 + T_3 (III) or T_3 (IV). Respective values in arbitrary units are listed in Table 1. Abbreviations used: CORT., corticosterone; rRNA, 28 S ribosomal RNA.

Table 2 Synergy between PACAP and T_3 on CBG mRNA levels

Results, expressed in arbitrary units for 15 μg of total RNA, are means $\pm$ S.E.M. for data from the Northern blot shown in Figure 5. Statistical significance levels: ***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$ compared with control cells.

Treatment	CBG mRNA
None	27.7 ± 4.2
PACAP	$239.3 \pm 24.0^{***}$
PACAP + T_3	$1078.0 \pm 59.3^{***}$
T_3	$136.1 \pm 16.3^*$

Up-regulation by dbcAMP of CBG expression

Next, because PACAPs transduce in hepatocytes mainly through the adenylate cyclase pathway, we challenged the cells with dbcAMP to determine whether the cell-permeant cAMP analogue would mimic the effect of the neuropeptide. Incubation of hepatocytes with dbcAMP actually elicited a dose-dependent elevation of CBG binding capacity, with half-maximal and maximal effects being achieved at about 25 and 100 nM (Figure 6). As calculated from saturation binding experiments (Figure 4), enhancement by dbcAMP of CBG binding was actually due to

Figure 6 Stimulatory effect of dbcAMP on CBG expression in cultured hepatocytes: concentration of binding sites and CBG mRNA levels

Cells were exposed, or not, to graded doses of dbcAMP for 4 days, as detailed in the legend to Figure 5. Maximum binding was expressed in terms of d.p.m. of labelled corticosterone bound per well. Bars are means $\pm$ S.E.M. for six culture wells, done in triplicate. Significance values: *** $P < 0.001$ and ** $P < 0.01$ compared with controls, in the absence of dbcAMP. At the top is shown a typical autoradiograph of a Northern blot for RNA extracts of control (Ctr.) cells and cells treated with 0.1 mM dbcAMP (cAMP). Respective values in arbitrary units (per 15 μ g of total RNA) were: 21.7 ± 5.5 and 53.4 ± 7.5 . Other abbreviations are as in the legend to Figure 5.

an increase in binding capacity, without a marked change in the value of the apparent dissociation constant ($K_d 2.0 \pm 0.3$ nM compared with a control value of 1.4 ± 0.2 nM). Here again, this effect could not be accounted for by mere augmentation of cell density, as there was no significant difference in DNA concentrations between control and treated cells (23.5 ± 0.5 μ g per well compared with 26.0 ± 0.4 μ g per well respectively).

Moreover, in a similar manner to the effect of PACAP, dbcAMP also increased CBG mRNA levels significantly ($P < 0.01$) from 21.7 ± 5.5 to 54.3 ± 7.1 arbitrary units, in control cells and in cells treated with dbcAMP respectively (Figure 6).

Synergistic effect between PACAP and T_3

Because we recently demonstrated [10] a direct stimulatory influence of T_3 on CBG biosynthesis in cultured hepatocytes, we wished to test the cells for possible cross-talk between the effect of the thyroid hormone and that of PACAPs. As depicted in Figure 5, we found that either PACAP38 or PACAP27 at 10 nM was able to stimulate an approx. 3-fold elevation of CBG's B_{max} ($P < 0.001$ compared with control). Interestingly, it seemed that on exposure of hepatocytes to both PACAP and T_3 there was a dramatic increase (about 7 times the basal value) in CBG binding, which was greater than would have been expected from mere additivity of individual effects.

Co-incubation of hepatocytes with PACAP together with T_3 likewise caused an amplification of CBG mRNA levels (Figure 5

and Table 2). The latter amounted to about 3 times the value that would have been reached in the case of mere additivity of individual effects.

Finally, we show that the synergistic influence of T_3 and PACAP on CBG expression could not be explained on the basis of a parallel enhancement of cAMP production. There was no significant change in hepatocytes incubated with PACAP together with T_3 , compared with PACAP alone (Table 1).

DISCUSSION

The present study clearly demonstrates the presence of functional receptors for PACAP in cultured fetal hepatocytes, the activation of which stimulates cAMP formation and CBG gene expression. Because the peptide's effect was mimicked on an equimolar basis by VIP, and PACAP failed to activate phosphoinositide breakdown, it can be concluded that the receptors called into play in these cells have the main characteristics of type II or type PVR2 isoforms, as recently coined by Rawlings et al. [5]. This finding on fetal hepatocytes confirms previous evidence based on PACAP and VIP binding to membranes of adult hepatocytes [6,8]. However, the failure of PACAP to elevate the $\text{Ins}(1,4,5)P_3$ level and, in turn, to release intracellular stores of Ca^{2+} does not necessarily preclude involvement of the peptide in the regulation of intracellular Ca^{2+} levels. Indeed, calcium influx could be indirectly triggered by cAMP, most probably via channel phosphorylation through cAMP-dependent protein kinase [23–25].

The stimulatory influence of PACAP on CBG biosynthesis is achieved with EC_{50} values close to the nanomolar range of values reported for the receptor's dissociation constant in various tissues as well as in liver membranes [1,4,6]. However, clear-cut demonstration of this stimulatory influence required hepatocytes to be exposed chronically to PACAP, as already seen for the effects of retinoic acid and T_3 [10], as well as under experimental conditions *in vivo*, after injection of rats with either thyroxine or glucocorticoids, i.e. treatments that enhanced and thwarted CBG production respectively [18,26]. Although long-term exposure of cells to ligands is generally associated with the down-regulation of receptor-effector complexes [27,28], it seemed that in fetal hepatocytes PACAP only partly dampened cAMP production. This could be due to the functional immaturity of fetal hepatocytes with regard to the desensitization process, which is a complex phenomenon that calls into play various phosphorylation and dephosphorylation reactions as well as molecules such as arrestins and receptor kinases [28–30].

An interesting finding in the present study is the demonstration of a synergistic interaction between T_3 and PACAP in the regulation of CBG biosynthesis. This type of interaction between hormone effects triggered at the level of nuclear receptors and that of cell membrane receptors has been observed before. In pituitary cells, for example, glucocorticoids and oestrogens modulate the gene expression of pro-opiomelanocortin and luteinizing hormone responses to gonadotropin-releasing hormone respectively [31,32]. Also, oestrogens have been shown to up-regulate thyrotropin-releasing hormone receptors in the pituitary [33]. Most interestingly, gene expression of tissue-type plasminogen activator in HTC hepatoma cells was similarly found to be regulated in a synergistic way by glucocorticoids and cAMP [34], whereas cAMP-induced induction of phosphoenolpyruvate carboxykinase in cultured hepatocytes was reported to require the presence of thyroid hormones [35]. In the present study, the synergistic enhancement of CBG synthesis by T_3 and PACAP could tentatively be explained by interactions between gene products triggered by T_3 and so-called transcription

Figure 7 Schematic model showing the putative influence of CBG on gluconeogenesis in hepatocytes

PACAP, by elevating cAMP levels, might play a dual role in regulating gluconeogenesis: a direct stimulatory effect and an indirect, opposite, one. We suggest the latter to be exerted via increased synthesis of CBG, which in turn (by favouring the formation of bound corticosterone) decreases the unbound, biologically active, form of the steroid. For the sake of clarity, the stimulatory effect of glucagon and the repression exerted by insulin on gluconeogenesis have not been depicted here. Also the presence of membrane receptors for CBG on hepatocytes has not been taken into account either. B, compound B or corticosterone.

factors, including cAMP response element (CRE) binding protein and CRE modulator, which modulate the activity of CRE in the gene promoter region [36,37]. That issue seems to be supported by recent findings that CRE binding protein might synergize with various transcription factors to modulate CRE activity in tumour hepatocytes [38]. However, such a mechanism of action is rather speculative here, because the presence of CRE in the promoter region of the CBG gene awaits demonstration in rat fetal hepatocytes.

In this context, one may also suggest that PACAP, by inducing cAMP formation in liver cells, could be involved in the regulation of a number of genes (which are under either the positive or the negative control of cAMP), as well as in the phosphorylation by cAMP-dependent protein kinase of various substrates [15–17,39,40]. Conversely, it is most likely that activation of adenylate cyclase activity by ligands such as glucagon and β -adrenergic agonists, for instance, would also impact, like PACAP, on CBG biosynthesis.

Receptors for PACAP are widely distributed in various areas outside the brain, where they appear to serve a series of selective biological functions [1,2]. In the pancreas, for instance, activation of PACAP-3 receptors by PACAP most probably released from nerve fibres [40] triggers release of insulin [41]. In the liver, PACAP stimulates glucose output from perfused tissue, although less potently than glucagon [42]. Given the well defined effects of cAMP and glucocorticoids on gluconeogenesis, a schematic model system may be put forward (Figure 7). In this system, PACAP, by enhancing CBG synthesis and in turn reducing unbound glucocorticoid levels, would participate in a subtle and

negative way in the regulation of gluconeogenesis. The circulating level of CBG indeed plays a crucial role in the extent to which the unbound moiety of glucocorticoids is transferred into the liver and hence in the biological activity of these steroids [43]. Moreover, the localization of receptors for CBG on cell membranes [13], and especially on hepatic membranes [44], together with the presence of CBG inside the cells (which is likely to interfere competitively with the glucocorticoid's access to the nucleus), confers additional degrees of complexity on the mechanisms of action of CBG at the cellular level. More work is clearly needed to clarify these issues.

Taken together with our previous findings [9–11], the present study indicates that CBG expression in cultured hepatocytes of fetal rats is regulated in a complex fashion. Thus thyroid hormones and PACAP both seem to increase on their own and synergize to enhance synthesis of CBG, whereas retinoic acids and 1,25-dihydroxyvitamin D₃, in addition to glucocorticoids [18], exert opposite effects. It remains, however, to be determined whether these agents actually alter CBG expression at the transcriptional and/or post-transcriptional levels and play a similar role in controlling CBG synthesis *in vivo*.

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