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# Lack of effect of calcitonin gene-related peptide and amylin on major markers of glucose metabolism in hepatocytes

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**Abstract**

Effects of amylin and calcitonin gene-related peptide on several processes involved in carbohydrate metabolism were investigated in rat hepatocytes, non-parenchymal cells (Kupffer, Ito and endothelial cells) and alveolar macrophages. In hepatocytes, cAMP levels were increased 25-fold by glucagon (10 nM), less than 2-fold by calcitonin gene-related peptide (100 nM) and not at all by amylin (100 nM). In non-parenchymal cells and cultured alveolar macrophages, calcitonin gene-related peptide potently, and amylin weakly, stimulated cAMP levels. In hepatocytes neither amylin nor calcitonin gene-related peptide affected glycogen phosphorylase activity, glucose output, lactate uptake, glycogen synthesis, glycogen mass or tyrosine aminotransferase activity. The density of calcitonin gene-related peptide specific binding sites in parenchymal cells was 10-fold less then seen in non-parenchymal cells. We found no significant evidence of specific amylin binding sites. These results are consistent with the notion that amylin does not exert a direct effect in hepatocytes. However, we do not rule out that amylin may affect hepatic glucose output indirectly through Cori cycling of lactate derived from skeletal muscle or from interactions through non-parenchymal cells.

*Keywords:* Amylin; CGRP (calcitonin gene-related peptide); Hepatocyte; Glucose metabolism; Macrophage; Kupffer cell

## 1. Introduction

Amylin is a 37 amino acid polypeptide synthesized by and secreted from the β-cells of the pancreas along with insulin, and which shares approximately 50% structural homology with the neuropeptide calcitonin gene-related peptide (Leighton and Cooper, 1988; Cooper, 1994; Rink et al., 1993; Pittner et al., 1994). Amylin appears to serve as a partner to insulin in regulating blood glucose, in part via potent regulatory effects on uptake of glucose from food by modulation of gastric emptying (Young et al., 1995a,b).

Amylin also has well documented effects on intermediary carbohydrate metabolism in rodents in vivo. The available data strongly support the idea that amylin acts on rodent skeletal muscle to increase cAMP levels (Pittner et al., 1995), activate glycogen phosphorylase (Young et al., 1991b; Deems et al., 1991) and glycogenolysis (Young et al., 1990) and stimulate lactate production in isolated soleus muscle preparations (Leighton and Cooper, 1988; Pittner et al., 1995) as well as from an in situ rat hindlimb preparation (Vine et al., 1995). Administration of pharmacological concentrations of amylin into rats rapidly increases plasma lactate concentration (Young et al., 1991c), shown in hyperlactemic clamp experiments to be due to increased lactate release (Rink et al., 1994), which, through Cori cycling, results in an increase in hepatic glucose production. It has been reported that this hyperglycemic effect of amylin observed in fasted rats is more potent than that seen with glucagon (Wang et al., 1991), and is mimicked by CGRP (Choi et al., 1991; Rossetti et al., 1993; Young et al., 1993; Molina et al., 1990). Regulation of the supply of lactate from muscle may be the main or the only way in which amylin promotes hepatic glucose production, but the available in vivo data do not rule out an additional effect of amylin on the liver itself.

Reports addressing direct actions of amylin or calcitonin gene-related peptide in liver present a confusing picture. In rodents, amylin has been said to increase apparent hepatic glucose output in both euglycemic clamp experiments (Molina et al., 1990; Koopmans et al., 1991) and under non-clamped conditions (Young et al., 1991c). How-

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ever, the hyperglycemic effects of amylin reportedly can be mimicked by replicating amylin-induced hyperlactemia with lactate infusions (Young et al., 1991c), raising the question whether amylin-mediated glucose production is related primarily to its lactate-mobilizing actions in skeletal muscle (which increase gluconeogenic substrate supply), or whether amylin directly affects the liver.

Calcitonin-gene-related peptide and amylin are reported to stimulate adenylate cyclase in liver membranes, the effects of amylin being 100–1000-fold less potent than calcitonin gene-related peptide, a ratio consistent with action through calcitonin gene-related peptide receptors (Zhu et al., 1991; Poyner et al., 1992; Bushfield et al., 1993; Morishita et al., 1990). However, amylin binding appears to be restricted to non-parenchymal cells and may not be associated with hepatocytes at all (Stephens et al., 1991). Despite the evidence supporting amylin stimulation of endogenous glucose production in intact rats, amylin is reported to not increase glucose output in the perfused liver (Nishimura et al., 1992; Roden et al., 1992) or in isolated hepatocytes (Stephens et al., 1991; Morishita et al., 1992). Nor did amylin reportedly affect glucagon- or insulin-induced changes in glucose production from perfused liver (Nishimura et al., 1992; Roden et al., 1992) or isolated hepatocytes (Stephens et al., 1991) or insulin-induced changes in glucokinase expression in hepatocytes (Nouspikel et al., 1992). These findings are all consistent with the notion that amylin has no direct effect on hepatocytes.

In contrast to this interpretation are several studies that report direct effects of calcitonin gene-related peptide and amylin in hepatocytes, including weak stimulation of cAMP levels and glycogen phosphorylase *a* activity in hepatocytes in the presence of phosphodiesterase inhibitors (Bushfield et al., 1993), stimulation of lipogenesis (Suzuki et al., 1992), stimulation of glycogenolysis (Suzuki et al., 1992; Ciaraldi et al., 1992) and stimulation of gluconeogenesis from pyruvate (Ciaraldi et al., 1992). In other studies amylin and calcitonin gene-related peptide reportedly inhibited the ability of insulin to stimulate glycogen synthase and glycogen synthesis, inhibit glucagon-activated glycogen phosphorylase and inhibit phosphoenolpyruvate carboxykinase mRNA expression (Gómez-Foix et al., 1991; Baque et al., 1994). Taken together these reported effects of amylin in hepatocytes are either glucagon-like (Bushfield et al., 1993; Nouspikel et al., 1992; Suzuki et al., 1992), insulin-like (Suzuki et al., 1992) or anti-insulin-like (Gómez-Foix et al., 1991; Baque et al., 1994) and occur at 1 pM (Ciaraldi et al., 1992), but not at 1–10 nM (Ciaraldi et al., 1992), 1–10 nM (Suzuki et al., 1992; Gómez-Foix et al., 1991; Baque et al., 1994) or only at concentrations above 100 nM (Bushfield et al., 1993).

In an effort to resolve some of these contradictory findings, we have separately examined the acute and chronic effects of amylin and calcitonin gene-related peptide on a number of processes relevant to carbohydrate metabolism, including tissue binding, cAMP generation, glycogenolysis and gluconeogenesis in freshly isolated or overnight primary cultures of rat hepatocytes, non-parenchymal cells (Kupffer cells and endothelial cells) and alveolar macrophages (which show many similarities to Kupffer cells). Particular care was taken to ensure the viability and responsiveness of the hepatocytes by measuring responses to insulin and glucagon. Our results show that calcitonin gene-related peptide and amylin (probably through calcitonin gene-related peptide receptors) have direct effects on non-parenchymal cells, but do not have a significant effects on any parameter measured in hepatocytes.

## 2. Materials and methods

### *2.1. Materials*

Rat amylin, rat calcitonin gene-related peptide α, rat and salmon calcitonin were from Bachem California (Torrence, CA, USA). Stock solutions of amylin were tested for peptide and amino acid content (Young et al., 1992). Recombinant human insulin (Humulin-R) was from Eli Lilly (Indianapolis, IN, USA). Other chemicals were of reagent grade from Sigma (St. Louis, MO, USA) or Fisher Scientific (Atlanta, GA, USA). D-[U-$^{14}$C]Glucose and [U-$^{14}$C]lactate were from Amersham and cAMP radioimmunoassay kit was from Biomedical Technologies (Stoughton, MA, USA). Tissue culture materials were from Gibco BRL (Grand Island, NY, USA). Type 2 collagenase was from Worthington Biochemicals (Freehold, NJ, USA).

### *2.2. Isolation and culture of hepatocytes*

Hepatocytes were isolated as previously described (Berry and Friend, 1969; Pittner et al., 1985). Following collagenase digestion the cell suspension was centrifuged at $20 \times g$ for 2 min. Hepatocytes resuspended from the pellet were typically 90–95% viable, as judged by their ability to exclude trypan blue, and were microscopically judged to be $> 99\%$ free of non-parenchymal cells. Hepatocytes were plated in a modified Leibowitz L-15 medium containing either 10% (v/v) newborn-calf serum or 10% fetal-calf serum as described (Pittner et al., 1985) and incubated at 37°C in humidified air for 1 h before the unattached non-viable cells were removed by replacing the medium. Hepatocytes were either used within 4–6 h or after 2 h incubation in fresh medium if maintained overnight.

### *2.3. Isolation and culture of non-parenchymal cells*

Supernatants remaining after centrifugation of hepatocytes were pooled and centrifuged at $600 \times g$ for 10 min

to pellet material containing non-parenchymal cells. Kupffer and endothelial cells were isolated from Ito cells and hepatocyte cell debris using discontinuous two-layer (11.5 and 17.2%) Nycodenz density gradients as previously described (Hendriks et al., 1985). Cells on top of the 11.5% Nycodenz were highly enriched in Ito cells, whilst cells on top of the 17.2% interface consisted of a mixture of Kupffer and endothelial cells. Pure populations of either of these cells can only be obtained by the use of centrifugal elutriation (Spitzer and Pittner, 1993). The cells were microscopically judged to be free of intact parenchymal cells, however, we cannot exclude the possibility of subfractions (blebs) of parenchymal cells being present, resulting from the isolation procedure. Cells were washed and resuspended in Dulbecco's modified Eagle culture medium containing 10% fetal calf serum, plated at a density of $1 \times 10^6$ cells/well in 18 mm dishes (12-well plates) and incubated at 37°C in 5% $CO_2$ atmosphere. Unattached cells were removed by replacing the medium after 1 h. Cells were cultured for at least 3–4 h before use.

### 2.4. Isolation and culture of alveolar macrophages

Alveolar macrophages were isolated as described previously (Mayer et al., 1993) except a hypotonic shock of the cell pellet, to remove red blood cells, was not performed as the lungs were perfused prior to lavage. The yield of cells was increased by massaging the lungs during the lavage. Cells were resuspended in Dulbecco's modified Eagle medium and cultured as described above for non-parenchymal cells. Cells were $>90\%$ viable with $>95\%$ of cells being macrophages as determined using Wright stain. Cells were cultured for at least 4 h prior to use.

### 2.5. cAMP accumulation

Cells from 12-well plates (750 000 cells/well) were incubated for 20 min with Dulbecco's phosphate-buffered saline containing 5.5 mM glucose and 0.1% bovine serum albumin, pH 7.4 at 37°C in humidified air incubator, and a further 10–15 min in fresh buffer containing 0.5 mM 3-isobutyl-1-methylxanthine. Hormones were then added as indicated and incubations continued for an additional 20 min. Reactions were terminated by the addition of 10% trichloroacetic acid (TCA) after which the medium was neutralized with 0.8 M Trizma-base. cAMP in the supernatant was determined either by radioimmunoassay (BTI) or by scintillation proximity assay (Amersham). Results are expressed as pmol cAMP/well per 20 min.

### 2.6. Glycogen phosphorylase

Glycogen phosphorylase *a* in hepatocytes from 12-well plates (750 000 cells/well) was measured as described previously (Pittner and Spitzer, 1993) and determined from the rate of glucose 1-phosphate incorporation into glycogen as measured by the release of $P_i$ and results are expressed as pmol $P_i$ produced/min per well.

### 2.7. Glycogen mass

Hepatocytes in 60 mm tissue culture plates ($2 \times 10^6$ cells) were incubated for 1 h in Krebs-Ringer bicarbonate (KRB) buffer containing 0.1% bovine serum albumin, 5.5 mM glucose and hormones as indicated at 37°C in humidified 5% $CO_2$ atmosphere. Cells were then digested with 1 ml of 1 M KOH and glycogen in the samples was separated by ethanol precipitation of KOH digests (Young et al., 1992). Glycogen mass in precipitates was determined by measuring the glucose released from samples incubated with amyloglucosidase as described in detail previously (Young et al., 1991a). Protein was determined in parallel cultures by the method of Bradford (1976). Results are expressed as mg glycogen/g protein.

### 2.8. Glycogen synthesis and gluconeogenesis from lactate

Hepatocytes in 6-well plates ($1.25 \times 10^6$ cells/well) were incubated for 60 min at 37°C in KRB buffer containing 0.2% bovine serum albumin and 5 mM [$^3$H]lactate and 0.5 mM pyruvate, pH 7.4 containing hormones as indicated. [$^3$H]Glycogen was extracted by ethanol precipitation as described above and its formation is expressed as nmol [$^3$H]lactate incorporated/well per 60 min. [$^3$H]Glucose in the medium was separated from [$^3$H]lactate by ion exchange chromatography using Dowex AG 1X8 (3 ml, 200–400 mesh, formate form). Glucose was eluted with 6 ml of $H_2O$ and lactate was eluted with 6 ml of 1 M ammonium formate solution. Recovery of glucose was $>99\%$ in $H_2O$ containing $\leq 0.6\%$ of lactate and the recovery of lactate was $>99\%$ in formate containing $\leq 0.3\%$ of glucose. Medium not exposed to cells was used as a blank. Formation of labelled glucose from labelled lactate was expressed as nmol/well.

### 2.9. Lactate flux measurements

Hepatocytes in 6-well plates ($1.25 \times 10^6$ cells/well) were incubated overnight (20 h) in Leibowitz medium containing 10% serum and indicated hormones after which the medium was collected and centrifuged at $600 \times g$ for 10 min to remove any cells. Lactate and glucose in the supernatant were determined on an analyzer (L-lactate oxidase assay and glucose oxidase immobilized enzyme chemistries, Analyzer model 2300-STAT, YSI, Yellow Springs, OH, USA).

### 2.10. Tyrosine aminotransferase activity

Tyrosine aminotransferase is involved in the breakdown of amino acids whose carbon skeletons are used for gluconeogenesis. Like phosphoenolpyruvate carboxykinase, the

hormonal regulation of its synthesis is well documented, making it a good marker for chronic changes in gluconeogenic flux. Hepatocytes were maintained for 20 h prior to the addition of hormones, following which, they were further incubated for 6 h as indicated. Tyrosine aminotransferase activity was measured from the rate of conversion of *p*-hydroxyphenylpyruvate from α-ketoglutarate. *p*-Hydroxyphenylpyruvate was converted to *p*-hydroxybenzaldehyde by strong alkali, and detected by absorbance at 331 nm (Diamondstone, 1966). Cells in 12-well plates ($0.75 \times 10^6$) were scraped in 0.5 ml of ice-cold sucrose buffer (0.25 M sucrose containing 0.5 mM dithiothreitol, 50 μM pyridoxal phosphate and 10 mM HEPES, pH 7.4) (Pittner et al., 1985), and frozen in liquid $N_2$. To assay tyrosine aminotransferase, samples were incubated with buffer containing 0.2 M potassium phosphate, 6 mM tyrosine, 4 mM diethylthiocarbamate (to inhibit *p*-hydroxyphenylpyruvic acid oxidase) and 37.5 μM pyridoxal phosphate, pH 7.4. Reactions were initiated with α-ketoglutarate (final concentration of 10 mM) continued for 10 min at 37°C, and terminated with 10 M NaOH. Absorbance at 331 nm was then determined. Reactions were linear up to 45 min. Results are expressed as nmol *p*-hydroxybenzaldehyde formed/min per mg protein.

### *2.11. Binding densities of calcitonin gene-related peptide, amylin and salmon calcitonin in hepatocytes and non-parenchymal cells*

Parenchymal cells, Ito cells or a mixed population of Kupffer cells and endothelial cells were resuspended at approximately $2–5 \times 10^6$ cells/ml in a modified KRB containing 120 mM NaCl, 5.4 mM KCl, 1.2 mM $KH_2PO_4$, 1.2 mM $MgSO_4$, 4.2 mM $NaHCO_3$, 25 mM Hepes, 20 mM glucose, 1 mg/ml bovine serum albumin and 0.5 mg/ml bacitracin at pH 7.4. Cells (50 μl) were incubated in 96-well plates in buffer containing either 22–31 pM (2-[$^{125}$I]iodohistidyl$^{10}$) human calcitonin gene-related peptide-1, 14–18 pM [$^{125}$I]BH-rat amylin or 16–35 pM [$^{125}$I]iodotyrosyl-salmon calcitonin for 2 h at 4°C and then filtered through glass fiber filters to determine total binding (Kenney et al., 1993). Non-specific binding was measured in the presence of $10^{-6}$ M unlabelled peptide. Protein was determined by the method of Bradford (1976) with bovine serum albumin as standard.

### *2.12. Competition of $^{125}$I-calcitonin gene-related peptide binding to hepatocytes*

Parenchymal cells in 6-well culture plates were incubated in 1 ml of modified Krebs-Ringer buffer, as described above, containing 27.8–28.4 pM (2-[$^{125}$I]iodohistidyl$^{10}$) human calcitonin gene-related peptide-1 and unlabelled peptides as described for 2 h at 4°C. Plates were then washed three times with 2 ml of ice-cold phosphate-buffered saline and incubated at 4°C overnight in 1 ml of 1 M NaOH to digest the cells. Radioactivity was determined in each well and binding is expressed as fmol/mg protein.

Table 1
Specific binding densities of calcitonin gene-related peptide, amylin and salmon calcitonin to parenchymal and non-parenchymal cells of the liver

| | Calcitonin gene-related peptide binding | Amylin binding | Salmon calcitonin binding |
|---|---|---|---|
| Hepatocytes | 0.37 ± 0.08 | N.D. | N.D. |
| Ito cells | 2.3 ± 0.4 | < 100 cpm | N.D. |
| Kupffer/endothelial cells | 3.2 ± 0.4 | < 100 cpm | N.D. |

Binding densities in hepatocytes, Ito cells and a mixed population of Kupffer cells and endothelial cells were determined as described in Section 2. Specific binding is expressed as fmol/mg protein and results are means ± S.E. from 3–5 independent experiments. N.D. means not detected.

## 3. Results

### *3.1. Binding studies*

Specific binding sites for calcitonin gene-related peptide were found in both parenchymal and non-parenchymal cell fractions (Table 1). The density of these binding sites in non-parenchymal cells was approximately 10× greater than that found in hepatocytes. There was no significant specific amylin or salmon calcitonin binding identified in any of the cell types studied. Calcitonin gene-related peptide (795 pM) was approximately 500× more potent than amylin (1.38 μM) in displacing bound [$^{125}$I]calcitonin gene-related peptide from hepatocytes (Fig. 1). Salmon calcitonin did not appear to displace calcitonin gene-related peptide binding.

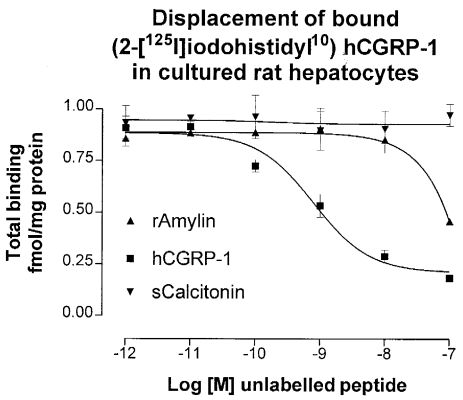


Fig. 1. Displacement of $^{125}$I-human calcitonin gene-related peptide-1 binding in 4-h cultured hepatocytes. Competition of binding of 28 pM $^{125}$I-human calcitonin gene-related peptide-1 to cells was measured in the presence of calcitonin gene-related peptide, amylin and salmon calcitonin at the indicated concentrations. Results are means ± S.E. from 3 independent experiments, measured in triplicate.

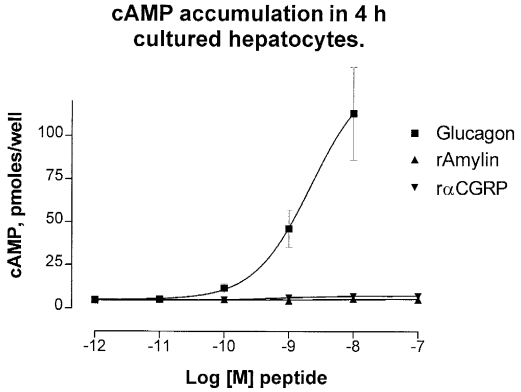


Fig. 2. Accumulation of cAMP in 4-h cultured hepatocytes. Cells were incubated for 20 min with either glucagon, amylin or calcitonin gene-related peptide at the concentrations indicated, in the presence of 0.5 mM 3-isobutyl-1-methylxanthine. cAMP content of the cells was determined by radioimmunoassay. Results are means ± S.E. from 2–3 independent experiments and are expressed as pmol cAMP/well.

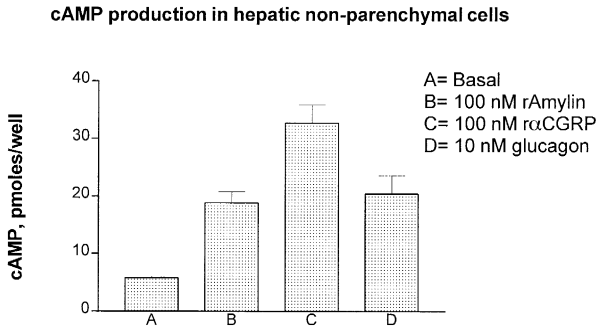


Fig. 3. Accumulation of cAMP in cultured non-parenchymal cells. Cells were incubated as described in the legend to Fig. 2. Results are means ± ranges from 2 independent experiments and are expressed as pmol cAMP/well.

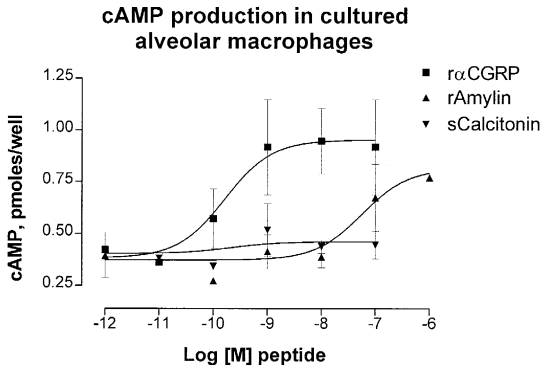


Fig. 4. Accumulation of cAMP in cultured alveolar macrophages. Cells were incubated as described in the legend to Fig. 2. Results are means ± S.E. from 2–3 independent experiments and are expressed as pmol cAMP/well.

### 3.2. Cyclic AMP generation

Neither amylin nor salmon calcitonin (not shown) at up to 100 nM significantly increased cAMP levels in hepatocytes. In contrast, glucagon potently stimulated cAMP generation 25-fold, with an $EC_{50}$ of 2.1 nM (Fig. 2). Calcitonin gene-related peptide (100 nM), only in the presence of the phosphodiesterase inhibitor 3-isobutyl-1-

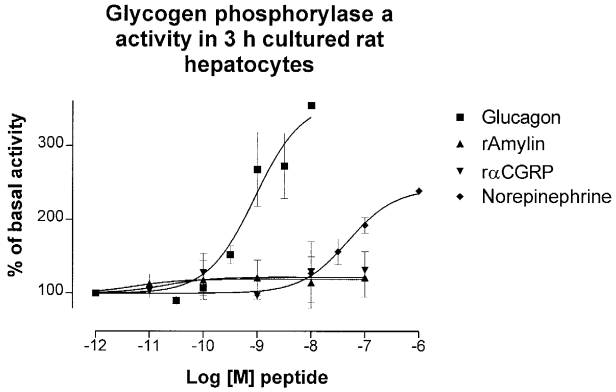


Fig. 5. Activation of glycogen phosphorylase in 3-h cultured hepatocytes. Cells were incubated for 2 min with glucagon, amylin, calcitonin gene-related peptide or epinephrine at the concentrations indicated in the absence of added phosphodiesterase inhibitor. Results are means ± S.E. from 3 independent experiments.

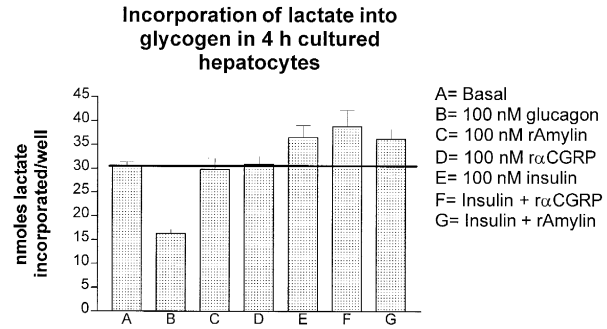


Fig. 6. Lactate incorporation into glycogen in 4-h cultured hepatocytes. Cells were incubated for 1 h with 5 mM glucose, 5 mM labelled lactate and 0.5 mM unlabelled pyruvate in the presence of glucagon, amylin, calcitonin gene-related peptide or insulin at the concentrations indicated. Incorporation of lactate into glycogen was determined. Results are means ± S.E. from 6 independent determinations and are expressed as nmol lactate incorporated into glycogen/well.

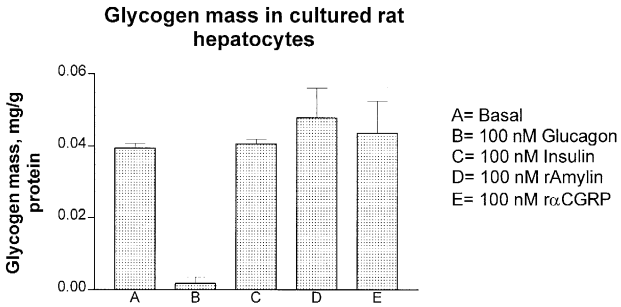


Fig. 7. Glycogen mass in 4-h cultured hepatocytes. Cells were incubated for 1 h in the presence of 100 nM glucagon, 100 nM insulin, 100 nM amylin or 100 nM rα calcitonin gene-related peptide and the glycogen content of the cells was then determined. Results are means ± ranges from 2 independent experiments measured in triplicate and are expressed as mg glycogen/g total protein.

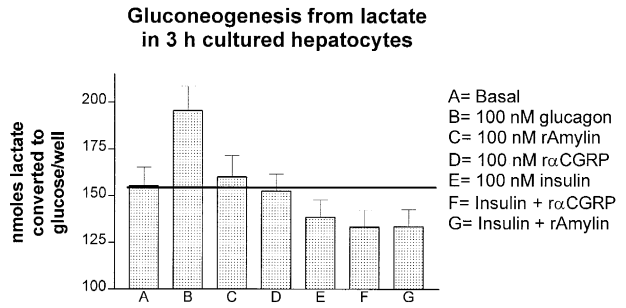


Fig. 8. Glucose output from labelled lactate in 4-h cultured hepatocytes. Labelled glucose appearing in the medium of cells incubated as described in the legend to Fig. 6 was determined by ion-exchange chromatography. Results are expressed as nmol lactate converted to extracellular glucose/well.

methylxanthine, increased cAMP levels 1.7-fold in hepatocytes ($7.83 \pm 0.86$ vs. $4.45 \pm 0.38$ pmol cAMP/well ($P < 0.05$, $n = 3$). Similar results were also seen with 20-h cultured hepatocytes that were allowed to fully recover from isolation following collagenase digestion (results not shown).

In non-parenchymal cells (mixed Kupffer and endothelial cells), calcitonin gene-related peptide (100 nM) increased cAMP levels by over 5-fold, compared to less than 3-fold by amylin at the same concentration (Fig. 3). Similar results were obtained in cultured alveolar macrophages which closely resemble Kupffer cells (Fig. 4); calcitonin gene-related peptide was 350-fold more potent than amylin in increasing cAMP levels ($EC_{50}$ 165 pM vs. 57 nM) and salmon calcitonin (up to 100 nM) had no effect.

### 3.3. Glycogen phosphorylase activation

Neither amylin, calcitonin gene-related peptide (Fig. 5) nor salmon calcitonin (results not shown), at up to 100 nM, significantly stimulated glycogen phosphorylase activity following a 2 min incubation in the absence of phosphodiesterase inhibitor. In contrast, glucagon ($EC_{50}$ 0.91 nM) and norepinephrine ($EC_{50}$ 49 nM) both potently stimulated glycogen phosphorylase activity (Fig. 5). Similar results were observed following 10 min, 20 min and 60 min of incubation and also in 20-h cultured hepatocytes (results not shown).

### 3.4. Lactate incorporation into glycogen

Glucagon (100 nM) inhibited by $47 \pm 3\%$ ($P < 0.001$, $n = 6$), and insulin (100 nM) increased by $20 \pm 8\%$ ($P < 0.05$, $n = 6$), the incorporation of labelled lactate into glycogen (Fig. 6). Neither amylin (100 nM) nor calcitonin gene-related peptide (100 nM) had any effect on label incorporated into glycogen either alone or in the presence of insulin (100 nM).

### 3.5. Glycogen mass

Glucagon decreased glycogen content of hepatocytes by $95 \pm 5\%$ following 1 h incubation (Fig. 7). Neither insulin, amylin or calcitonin gene-related peptide (all at 100 nM) under the same conditions had any measurable effect on glycogen mass.

### 3.6. Gluconeogenesis from lactate

In hepatocytes incubated with 5 mM [$^3$H]lactate and 5 mM unlabelled glucose, glucagon increased the release of labelled glucose by $26 \pm 8\%$ over 60 min, whereas insulin decreased glucose output by only $11 \pm 6\%$. Neither amylin nor calcitonin gene-related peptide had any effect on glucose output. Nor did they modify the ability of insulin to decrease glucose output (Fig. 8). When glucose concentration in the medium was raised to 20 mM, basal glucose output from labelled lactate fell to $61.1 \pm 5.7$ nmol/well (from $155.3 \pm 9.9$ nmol/well in the presence of 5 mM glucose). With 20 mM glucose in the incubation medium, glucagon approximately doubled glucose output to $123.1 \pm 5.9$ nmol/well, whereas insulin decreased glucose output approximately 30% to $46.4 \pm 16.3$ nmol/well. Under the same conditions, neither amylin nor calcitonin gene-related peptide affected glucose output either in the presence or absence of insulin (results not shown).

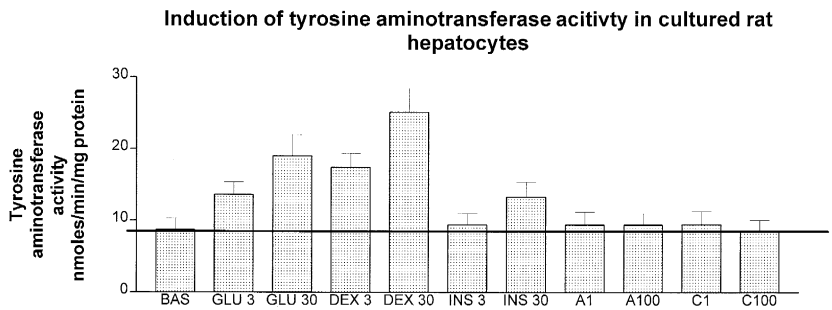


Fig. 9. Induction of tyrosine aminotransferase activity in 20-h cultured hepatocytes. Cells were incubated for 6 h in the presence of glucagon (GLU), dexamethasone (DEX), insulin (INS), amylin (A) or calcitonin gene-related peptide (C) at the nM concentration indicated. Tyrosine aminotransferase activity is expressed as nmol/min/mg protein and results are means ± S.E. from 4–5 independent experiments.

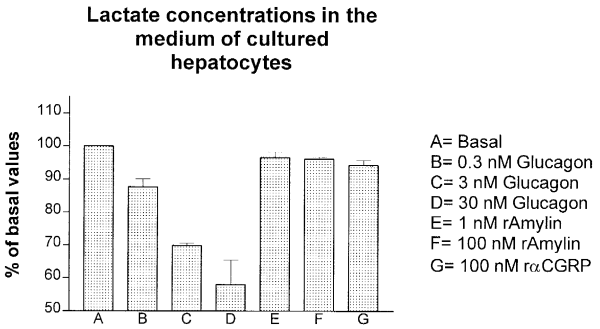


Fig. 10. Lactate concentration in medium of 20-h cultured hepatocytes. Cells were incubated for 20 h in the presence of glucagon, amylin or calcitonin gene-related peptide at the concentrations indicated after which the concentration of lactate in the medium was measured. Results are expressed as % of lactate compared to control incubations in the absence of added hormones. Results are means ± ranges from 2 independent experiments.

### *3.7. Tyrosine aminotransferase induction*

Glucagon (30 nM), dexamethasone (30 nM) and insulin (30 nM) each increased tyrosine aminotransferase activity by 119 ± 33%, 189 ± 38% and 53 ± 24%, respectively, over 6 h of incubation (Fig. 9). The changes seen were very similar to those previously reported (Pittner et al., 1985). Neither amylin nor calcitonin gene-related peptide up to 100 nM significantly induced tyrosine aminotransferase activity (Fig. 9). Nor did either peptide modify its induction by glucagon, dexamethasone or insulin (results not shown).

### *3.8. Lactate flux*

Glucagon decreased medium lactate concentrations up to 42 ± 7% over starting concentrations when incubated for 20 h with hepatocytes (Fig. 10). Effects of glucagon were observed at concentrations of 0.3 nM. Glucagon-mediated decreases in medium lactate were associated with a 9 ± 3% increase in medium glucose concentrations (Fig. 11). Amylin and calcitonin gene-related peptide at up to 100 nM had no significant effect on the concentrations of lactate or glucose in the medium.

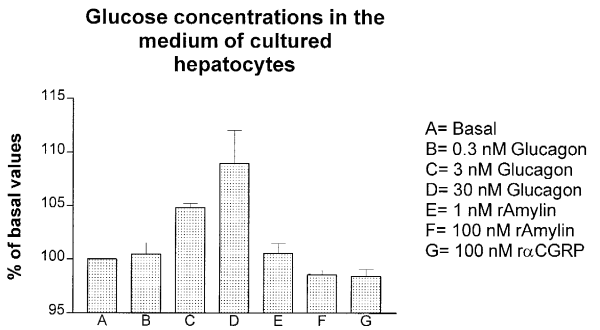


Fig. 11. Glucose concentration in medium of 20-h cultured hepatocytes. The concentration of glucose was determined in the medium of cells incubated as described in the legend to Fig. 10.

## 4. Discussion

Consistent with previous reports demonstrating calcitonin gene-related peptide binding sites in liver membranes, we found specific calcitonin gene-related peptide binding sites in hepatocytes (Morishita et al., 1990; Stephens et al., 1991). However, we found no physiologically relevant effect of calcitonin gene-related peptide, amylin or salmon calcitonin on cAMP levels, glycogenolytic fluxes, glucose output, gluconeogenic enzymes or fluxes in either acute or chronic studies of hepatocytes. For all of the parameters measured we showed clear effects of glucagon or insulin, demonstrating that the hepatocyte preparations were functionally viable, at least to these hormones.

We failed to observe previously reported glucagon-like (Suzuki et al., 1992; Ciaraldi et al., 1992) or anti-insulin-like (Gómez-Foix et al., 1991; Baque et al., 1994) effects of amylin or calcitonin gene-related peptide. Anti-insulin effects are reportedly dependent on the presence of fetal calf serum and disappear with other sera. We saw no difference in hepatocyte responses between cells incubated with fetal or new-born serum (results not shown). Glucagon-like effects of amylin have also been reported in the Hep G2 hepatoma cell line (Ciaraldi et al., 1992). We saw no effect of amylin on the insulin-responsive Hep G2 and H4-II-E hepatomas (results not shown).

The absence of effects of amylin and calcitonin gene-related peptide in hepatocytes in the present study is consistent with previous reports in hepatocytes (Bushfield et al., 1993; Stephens et al., 1991; Morishita et al., 1992; Nouspikel et al., 1992) and in perfused liver (Nishimura et al., 1992; Roden et al., 1992). From the binding data and the small increase in cAMP seen in the presence of 3-isobutyl-1-methylxanthine, it appears that hepatocytes contain calcitonin gene-related peptide receptors but are of insufficient number, or are improperly coupled to elicit a physiological response as seen, for example, with glucagon. On the other hand, we cannot exclude contaminating non-parenchymal cells being responsible for the small cAMP elevations observed after calcitonin gene-related peptide and, to a lesser extent, amylin.

Calcitonin gene-related peptide potently stimulated cAMP in non-parenchymal cells, while amylin and salmon calcitonin were either much less potent or were without effect. The non-parenchymal cells in these experiments consisted of a mixed population of Kupffer cells (resident macrophages), endothelial cells and possibly subfractions (blebs) of parenchymal cells (see Section 2.3, Isolation and culture of non-parenchymal cells). In purified alveolar macrophages which closely resemble Kupffer cells (Mayer et al., 1993; Vignery et al., 1991a; Owan and Ibaraki, 1994) and are free of liver parenchymal cell subfractions (blebs) and mostly free of endothelial cells ($< 5\%$), the pattern was similar; calcitonin gene-related peptide potently stimulated cAMP levels, whilst amylin was approxi-

mately 1000-fold less potent and salmon calcitonin was without effect. This order of potency is consistent with the action of calcitonin gene-related peptide receptors, consistent with the higher density of binding sites found on these cells, relative to hepatocytes (Table 1; Stephens et al., 1991) and is consistent with previously reported studies demonstrating binding sites for calcitonin gene-related peptide on peritoneal (Vignery et al., 1991b), bone-derived (Owan and Ibaraki, 1994) and alveolar (Vignery et al., 1991a) macrophages. Whilst a low amount of amylin binding (< 10% of calcitonin gene-related peptide binding) in non-parenchymal cells was previously demonstrated (Stephens et al., 1991), we did not find significant amylin binding sites in non-parenchymal cells in our studies. We cannot explain the difference in these results as similar methodologies were employed in both studies, with the exception that our binding studies were performed at 4°C as opposed to 15°C. However, our results are consistent with those of Galeazza et al. (1991) who could not demonstrate, under any conditions, amylin binding to liver plasma membranes.

Activation of non-parenchymal cells (Kupffer cells and endothelial cells) by calcitonin gene-related peptide or amylin could result in actions in parenchymal hepatocytes. Activated Kupffer cells are reported to secrete cytokines and prostaglandins that can stimulate glycogenolysis in hepatocytes (Casteleijn et al., 1988b; Brass and Garrity, 1990; Gómez-Foix et al., 1989), modify nitric oxide production (Spitzer and Pittner, 1993) or affect protein synthesis (West et al., 1989). Intercellular communication has been proposed as a mechanism mediating endotoxin effects on carbohydrate metabolism in the liver (Casteleijn et al., 1988a). However, the reported absence of amylin action in the perfused liver (Nishimura et al., 1992; Roden et al., 1992) suggests that such a mechanism is unlikely.

In conclusion, neither amylin nor calcitonin gene-related peptide had measurable effects on any parameter measured in hepatocytes, nor did either peptide affect responses of hepatocytes to insulin or glucagon. In contrast, calcitonin gene-related peptide and amylin (weakly) stimulated cAMP levels in non-parenchymal cells. We find no evidence to suggest that direct actions on hepatocytes are responsible for the hyperglycemic effects of amylin, calcitonin gene-related peptide and salmon calcitonin in rats. Further studies are required to determine whether amylin and calcitonin gene-related peptide may indirectly affect hepatocyte carbohydrate metabolism by stimulating cytokine, prostanoid or leukotriene secretion in hepatic non-parenchymal cells.

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