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Expression of glucagon-like peptide 1 receptor in a murine C cell line: Regulation of calcitonin gene by glucagon-like peptide 1

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Abstract We have characterized, by RT-PCR amplification using specific primers, the presence of glucagon-like peptide-1 (GLP-1) receptor mRNA in CA-77 cells, a C cell line derived from a rat medullary thyroid carcinoma. Down-regulation of the GLP-1 receptor mRNA was observed after exposure of CA-77 C cells with GLP-1 (7–37). Increased secretion of both calcitonin gene-related peptide (CGRP) and calcitonin (CT) occurred after treatment with GLP-1 (7–37) associated with elevated steady-state levels of CGRP and CT mRNA. GLP-1 (7–37) increased cAMP formation in CA-77 cells in a dose-dependent manner; exendin (9–39), a GLP-1 receptor antagonist, inhibited cAMP production. The GLP-1 peptide which is produced by intestinal cells could be involved in the control of CT secretion through an entero-thyroidal axis implying GLP-1 receptor and increased CT gene expression.

Key words: C-cell; Glucagon-like peptide 1; Glucagon-like peptide 1 receptor mRNA; Calcitonin mRNA; Calcitonin gene-related peptide mRNA

1. Introduction

Glucagon and glucagon-like peptide 1 (GLP-1) are structurally related peptides arising from the tissue-specific processing of preproglucagon. The precursor contains in addition to glicentin and glucagon the sequences of two glucagon-like peptides, GLP-1 and GLP-2, separated by an intervening sequence [1]. The posttranslational processing of preproglucagon differs in pancreas and intestine [2,3]. In the pancreas the precursor is processed to glucagon and GLP-1, and in both small and large intestines glicentin, GLP-1, GLP-2 and intervening peptide are found. Three different forms of GLP-1 are produced by intestinal L cells, GLP-1 (1–37), GLP-1 (7–36 amide), and GLP-1 (7–37), but only truncated forms of GLP-1 are biologically active [4] as potent insulin secretagogues. The existence of a gastroentero-thyroidal axis involved in the control of calcitonin (CT) secretion was postulated 23 years ago [5]. Glucagon [6] as well as partially purified enteroglucagon were shown to stimulate the CT secretion rate in pigs [5]. We have therefore studied the effects of GLP-1 (7–37) on the secretion of CGRP and CT by the CA-77 C cell line, and on the steady-state level of their mRNAs. The effect of GLP-1 (7–37) agonists and antagonists on cAMP production by CA-77 cells was analysed. We have also characterized by RT-PCR the presence of GLP-1 receptor mRNA, and showed a down-regulation of GLP-1 receptor mRNA induced by GLP-1 (7–37).

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2. Materials and methods

2.1. Cell culture

The CA-77 cells were maintained in DMEM/Ham's F10 (1:1) (Gibco/BRL, France) supplemented with 10 μg/ml insulin (Sigma), 3×10^{-8} M sodium selenite (Gibco/BRL, France), 5 μg/ml transferrin (Gibco/BRL, France), 110 mg/l sodium pyruvate (Gibco/BRL, France), and a mixture of antibiotics (100 units/ml penicillin+100 μg/ml streptomycin; Gibco/BRL, France). Cells were plated at an initial density of 4×10^4 cells/cm^2 in a medium containing DMEM/Ham's F10 (1:1), 10% heat-inactivated fetal calf serum (J. Boy, Reims, France), 110 mg/l sodium pyruvate, 100 units/ml penicillin, and 100 μg/ml streptomycin. After 48 h of plating, the medium was changed and replaced by the growth medium described above; the growth medium was changed at 48-h intervals. GLP-1 (7–37) from Peninsula Laboratories Europe Ltd (Merseyside, UK) was dissolved in 0.01 N HCl to 10^{-4} M and then in culture medium to reach 10^{-8} M. Dexamethasone (Sigma), was dissolved in absolute ethanol and then diluted in PBS to reach a final ethanol concentration of 0.005% in the growth medium. Controls with the same volume of vehicle were used in each case. Cells were harvested at confluence.

2.2. RNA extraction, and Northern blot hybridization

Total RNAs were extracted with guanidium thiocyanate method [7]. The RNA pellets were dissolved in sterile distilled water, and quantified by optical density at 260 nm. Aliquots of total RNAs were denatured using formaldehyde; they were electrophoresed on 1% agarose gel [8], and RNAs were transferred to Gene-screen membranes (NEN) using 0.025 M phosphate buffer (pH 6.5).

Hybridizations for the 3 probes (CT, CGRP, and cyclophilin) were performed under the same conditions. Membranes were prehybridized for 4 h at 42°C in 50% formamide, 0.2% polyvinyl pyrrolidone, 0.2% bovine serum albumin, 0.2% Ficoll, 0.05 M Tris-HCl (pH 7.5), 1 M NaCl, 0.1% sodium pyrophosphate, 1% sodium dodecyl sulfate (SDS), 10% dextran sulfate, and denatured salmon sperm DNA (100 μg/ml). Hybridization was carried out for 16 h at 42°C in a similar solution but depleted of NaCl and dextran sulfate. Labeled probes were added to the hybridization solution at 10 ng/ml; cDNA probes were denatured by heating (100°C for 10 min). Membranes were washed by two changes of 2×SSC (1×SSC = 0.1 M NaCl, and 0.015 M sodium citrate) at room temperature for 5 min, then 2 changes of 2×SSC, and 1% SDS at 55°C for 30 min, and finally 2 changes of 0.1×SSC at room temperature for 30 min. Dried membranes were exposed at −80°C to Kodak Royal X-Omat AR5 films. Autoradiographs were quantified in an automatic densitometer scanner (Shimadzu Scientific Instruments). For both probes (CT and CGRP) quantification involved a correction including the cyclophilin control probe, and thus our data were expressed as CT/cyclophilin ratios and CGRP/cyclophilin ratios.

2.3. Polymerase chain reaction

cDNA was synthesized from 3 μg of total RNA. The reaction mixture had a final volume of 20 μl, and contained 50 mM KCl, 20 mM Tris-HCl (pH 8.4), 2.5 mM $MgCl_2$, nuclease free BSA (1 mg/ml), 20 U of RNAsin, 200 U of M-MuLV reverse transcriptase (BRL), 1 mM of each dNTP and 50 pmol of a 3′ oligo dT primer. Annealing was performed for 10 min at 23°C and primer extension for 1 h at 37°C. The reaction was then diluted to 100 μl with the same buffer containing 50 pmol of each specific primer and 2.5 units of *Taq* polymerase. Amplification was performed during 30 cycles: 30 s at 95°C (denaturation), 30 s at 55°C (annealing) and 30 s at 72°C (ex-

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tension). Control reactions were performed as follows: positive control with total RNA extracted from CA-77 cells and negative ones with CA-77 cell total RNA in the absence of reverse transcriptase or after digestion with RNAase A (1 mg/ml) 1 h at 37°C.

2.4. Analysis of PCR products

Amplified products were analysed by electrophoresis in 1.2% agarose gel, visualized by ethidium bromide, transferred to Nylon membranes (NEN). The amplified cDNA was hybridized with a specific 48-mer oligonucleotide probe of the GLP-1 receptor cDNA [9] radiolabelled with [^{32}P]dCTP using Terminal Deoxynucleotidyl Transferase (NEN 3′ end labeling system). The membranes hybridized at 42°C in presence of 50% formamide were washed as described above.

2.5. Preparation of ^{32}P-labeled probes

The CT-specific probe was the *Bgl*II-*Nsi*I fragment of the human CT cDNA. The plasmid containing near full-length human CT cDNA was kindly provided by M.G. Rosenfeld (University of California San Diego, La Jolla, CA, USA). Competent XL-1 blue cells (Stratagene, La Jolla, CA, USA) were transformed by the pUC8 plasmid. Plasmid DNA was extracted using the alkaline lysis method and purified by ultracentrifugation on CsCl gradient. After enzymatic hydrolysis and electrophoresis, the fragment *Bgl*II-*Nsi*I was electroeluted from the agarose gel, and labeled with [^{32}P]dCTP by nick-translation. The CGRP-specific probe was the *Bam*II-*Eco*RI fragment of the human CGRP cDNA. The pUC8 plasmid containing the full length human CGRP cDNA was provided by M.G. Rosenfeld, and the probe was prepared as previously described for the CT probe. GLP-1 receptor insert was cloned in PGEM-T vector (Promega). The fragment of insert was PCR amplified using specific oligonucleotides primers separated by agarose gel electrophoresis and purified by elution using 'Jet Sorb System' (Bioprobe Systems) A cyclophilin mRNA probe was used to verify the specificity of the changes occurring in CT and CGRP mRNAs. A 48-mer oligonucleotide (Bioprobe System, Montreuil sous bois, France) specific for the cyclophilin mRNA was 5′ end-labeled using T_4 polynucleotide kinase and [γ-^{32}P]ATP, and then purified on Sephadex G-50 fine columns.

2.6. Calcitonin and CGRP contents

2.6.1. Tissue extraction. Cell culture medium was collected and treated as previously described [10] to measure the CT and CGRP levels. The peptide contents (CT and CGRP) of C cells were also measured by RIAs; therefore, 400 µl 0.1 N HCl were added to culture dishes. Then the cells were scraped off and sonicated for 15 s, allowed to stand for 16 h at 4°C, and then frozen at −35°C until assayed.

2.6.2. Radioimmunoassays (RIAs). The CT RIA was already reported [11,12]; in brief, the G813 antibody (goat antiserum raised against synthetic human CT) was a gift of Dr. H. Heath (Mayo Clinic and Mayo Foundation, Rochester, MN, USA), and the detection limit of the assay was 3.9 pg per tube. This assay used ^{125} I-labeled human CT and unlabeled synthetic human CT (Ciba-Geigy, Basel, Switzerland) as standard; therefore, the results are expressed as ng equivalent human CT/mg protein since the protein content of culture dishes was determined according to Lowry et al. [13]. Intra-assay variations for the CT RIA were 5% and inter-assay variations were 10%. For the CGRP RIA already described [14,15], we have used synthetic human CGRP (Sigma) as standard, a sheep antibody raised against synthetic human CGRP diluted 1:100000, and [^{125}I]iodohistidyl human CGRP from Amersham (Les Ulis, France). The tubes were preincubated for 4 days at 4°C and then incubated for 3 days at 4°C in the presence of labeled CGRP. The bound and free fractions were separated by adding 0.15 ml of dextran choarcal suspension buffer to each assay tube (0.5 ml); after centrifugation at 2000×*g* for 20 min, the supernatant was discarded. The detection limit of this assay was 10 pg per tube with intra-assay variations of 6%, and inter-assay variations of 11%.

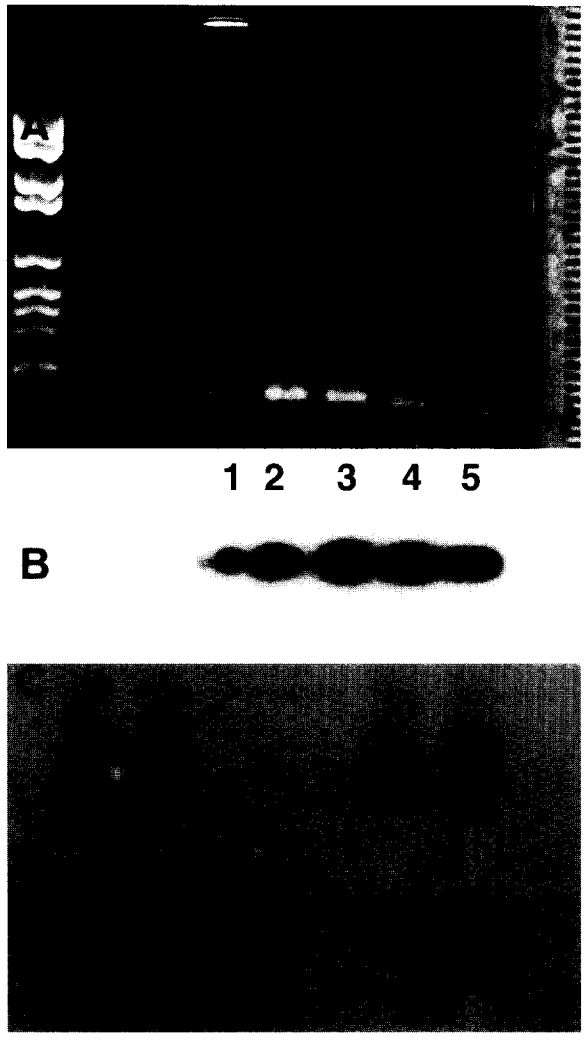

Fig. 1. (A) Agarose gel analysis of PCR products. Total RNAs extracted from CA-77 C cells were reverse transcribed and amplified with specific rat GLP-1 receptor primers. One quarter of the reaction was electrophoresed. The 1.2% agarose gel contained 2 mg/ml ethidium bromide. Lanes 1–5: 1.5, 3, 6, 9, 12 µg of total RNAs. (B) Autoradiogram of the Southern blot of PCR products (A) hybridized with ^{32}P-radiolabeled rat GLP-1 receptor-specific probe (see Table 1). (C) Northern hybridization of 25 µg total RNAs extracted from CA-77 C cells treated with 10^{-8} M GLP-1 (7–37), and hybridized with the 219 bp PCR product labeled with ^{32}P by random priming; the signal was observed after 4 days of exposure. Two mRNAs of 2.7 and 3.6 kb were detected (upper side). At the bottom, hybridization with a control cyclophilin probe. (C) Controls, and cells treated from 3 h.

2.7. Adenylyl cyclase activity assay

The production of cyclic AMP in CA-77 cells in the absence (con-

Table 1
Sequence and localisation of the primers used

Primers	Sequence 5′-3′
TGTACCTGAGCATAGGCTGG	819 Sense
TGAGATTAGCCTTCAGCTTGG	1034 Antisense
GCAAGCGTATGATGAGCCAATAGTTCATGTTGGAGTTCCTGGTCCAGC	Probe 903–950

The primers, and the probe were chosen from the cDNA sequence reported in [9].

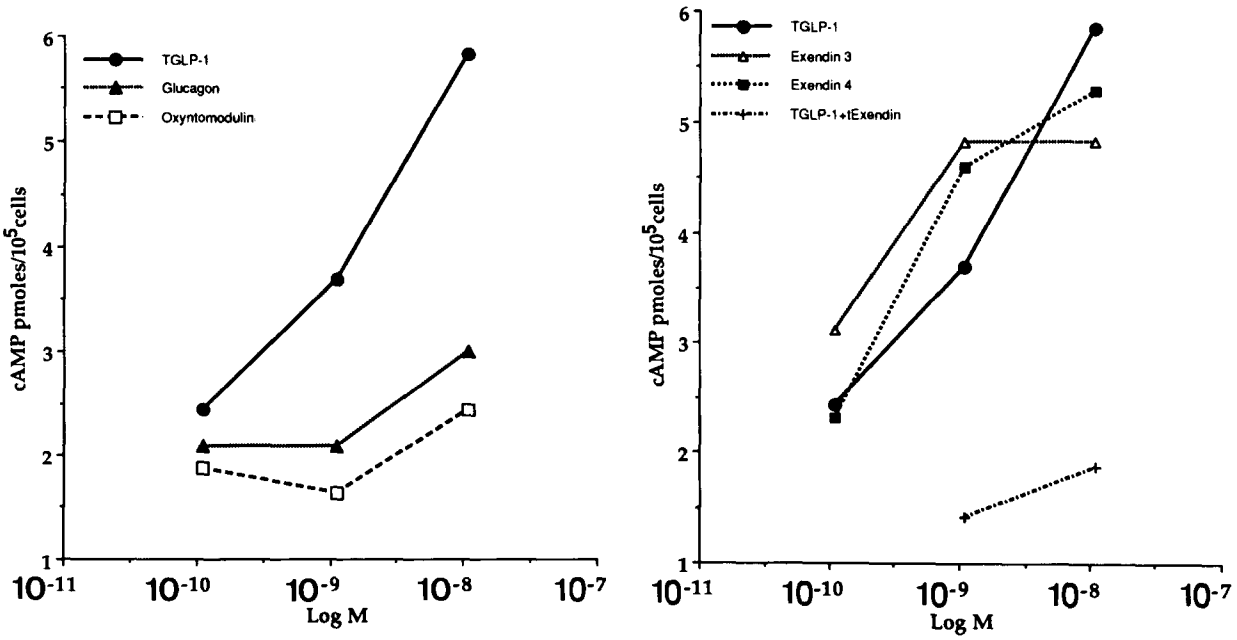

Fig. 2. Effects of GLP-1 (7–37) agonists and antagonists on cAMP production by CA-77 C cells. Each point was the mean of duplicates. The basal value was 0.68 pmol cAMP/10^5 cells. TGLP-1, GLP-1 (7–37); tExendin, exendin (9–39).

trol) or presence of different peptides was measured by RIA [16] from cells treated as previously described [17]. Porcine glucagon was obtained from Novo Research Institute (Bagsvaerdt, Denmark), and porcine oxyntomodulin was a gift from Dr. D. Bataille (INSERM Unit 376, Montpellier). Exendin-3, exendin-4, and exendin (9–39) were generously provided by Dr. J. Eng (VA Medical Center, Bronx, New York, USA).

2.8. Statistical analysis

All probabilities were calculated using the Mann-Whitney U-test for ranked non-parametric data. All data are presented as the mean ± S.E.M. of four determinations.

3. Results

3.1. Identification of GLP-1 receptor mRNA

The primers used for amplification of the PCR products are reported in Table 1. Gel electrophoresis of RT-PCR amplification products using specific GLP-1 receptor primers revealed the presence in CA-77 C cells of a single band stained by ethidium bromide (Fig. 1A). This band had the correct size of 219 bases and gave a strong signal after transfer and hybridization with a specific rat GLP-1 receptor probe (Fig. 1B).

No amplification was observed when the aliquots studied were treated with RNAase before reverse transcription and amplification (data not shown).

The presence of rat GLP-1 receptor mRNA in CA-77 C cells established by RT-PCR was substantiated by Northern blot. When 25 μg of total RNA and the 219 bp fragment obtained by PCR amplification was used as probe and labeled by random priming, a strong signal was observed after 4 days of exposure as two transcripts of 2.7 and 3.6 kb (Fig. 1C).

3.2. Biological effects of GLP-1 (7 to 37)

3.2.1. Effects on cAMP production. The GLP-1 (7–37) stimulation of adenylyl cyclase activity has been described as a receptor-mediated process requiring a functional coupling to GTP-binding proteins [18]. Thus, the effect of GLP-1 agonists on adenylyl cyclase activity in Ca-77 cells was investigated to ascertain the functionality of the GLP-1 receptor. The results in Fig. 2 show that all peptides tested with the exception of exendin (9–39) stimulated cAMP production. Maximal stimulation of adenylyl cyclase activity obtained with GLP-1 (7–37), glucagon, oxyntomodulin, exendin 3, and exendin 4 was 3.5–8.5-fold over basal values (0.68 pmol cAMP produced/10^5 cells). However, 10^{-8} M GLP-1 (7–37) was more efficient than the same dose of glucagon or oxyntomodulin. GLP-1 (7–37) and its agonists exendin-3 and exendin-4 were equipotent (Fig. 2). Exendin (9–39) at 10^{-6} M, a previously described antagonist of GLP-1 receptor, blunted the stimulation of cAMP production (Fig. 2) induced by GLP-1 (7–37).

3.2.2. Effects on both CGRP and CT secretion. CGRP secretion was clearly increased after short-time incubation of CA-77 cells with 10^{-8} M GLP-1 (7–37). A 2-fold increase in the CGRP release in the culture medium was regularly observed after 1 h of treatment (67.4 ± 7.8 vs. 32.8 ± 0.6 ng/mg protein in controls, $P < 0.01$), this rise being still maintained after 3 h of hormone exposure (Fig. 3). A similar response was observed for the CT secretion (332 ± 28 vs. 218 ± 12 pg/mg protein in controls after 3 h of treatment, $P < 0.05$). In all experiments performed, the C cell content of both CGRP and CT (data not shown) was reduced (−20%) after 3 h of exposure to 10^{-8} M GLP-1 (7–37) (Fig. 3). Sensitisation of C cell by previous exposure to 10^{-6} M dexamethasone for 20 h changed the induction of neither CT nor CGRP secretions after 1 h of treatment with 10^{-8} M GLP-1 (7–37) (412 ± 40 vs. 228 ± 32 pg CT/mg protein in controls, $P < 0.05$; and 195 ± 30 vs. 112 ± 22 ng CGRP/mg protein in controls, $P < 0.05$).

3.2.3. Effects on both CGRP and CT mRNA levels. An increase in the steady-state level of CGRP mRNA was observed from 3 to 48 h after treatment of CA-77 cells with 10^{-8} M GLP-1 (7–37); the ratio CGRP mRNA/cyclophilin mRNA was maximally increased in all experiments by a factor of 2

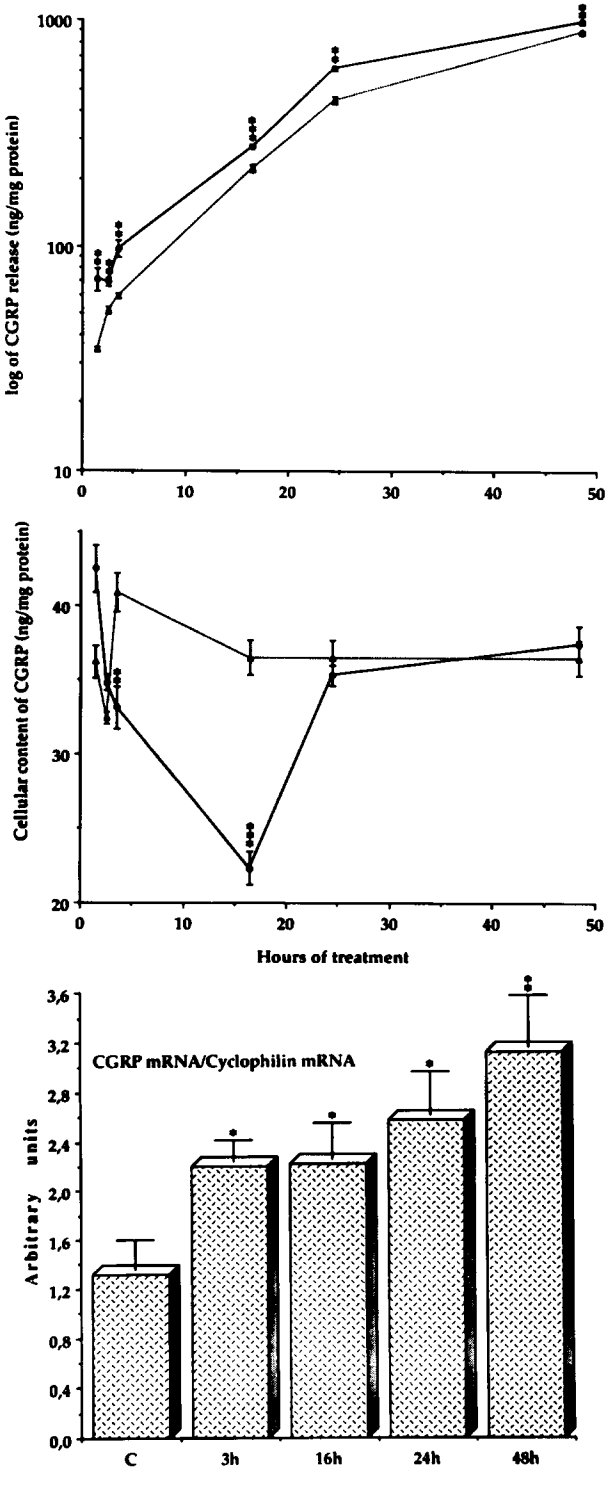

Fig. 3. Effects of 10^{-8} M GLP-1 (7–37) on CGRP secretion, cellular content of CGRP, and CGRP mRNA by CA-77 C cells. The results were expressed as the ratio CGRP mRNA/cyclophilin mRNA used as a control probe. Treated (———); controls (· · ·). Means ± S.E.M. of four culture dishes. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.01$ from controls (C).

(Fig. 3). As shown in Fig. 3, the increased CGRP and CT mRNAs (0.50 ± 0.08 vs. 0.17 ± 0.04 arbitrary units for CT mRNA in controls, $P < 0.05$) after 16 h of exposure to the hormone was associated with a reduction in the cellular content of both peptides (190 ± 15 vs. 475 ± 14 pg CT/mg protein

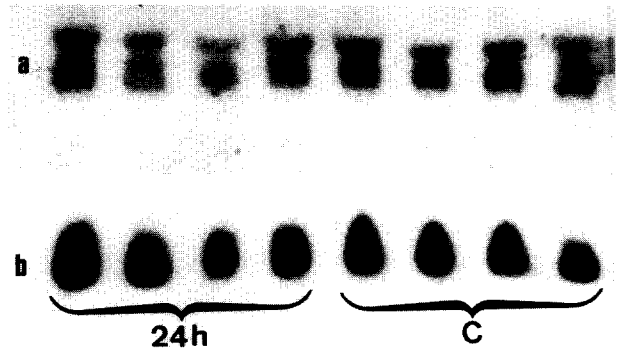

Fig. 4. Northern hybridization of 24 μg total RNAs extracted from CA-77 C cells treated for 24 h with 10^{-8} M GLP-1 (7–37). (a) Hybridization with the 219 bp specific GLP-1 receptor probe. (b) Hybridization with a control cyclophilin probe. (C) Controls.

in controls, $P < 0.001$) which can be accounted for by an elevated secretion of both peptides.

3.3. Variations in GLP-1 receptor mRNA

Exposure of C cells to 10^{-6} M dexamethasone elicited small changes in GLP-1 receptor mRNA (data not shown); a transient increase (+31% after 3 h) occurred followed by a reduction of its level (−28% after 48 h of treatment). Down-regulation of the rat GLP-1 receptor mRNA was observed after treatment of CA-77 C cells with 10^{-8} M GLP-1 (7–37). An 80% decrease was observed after 3 h of treatment as the ratio of the GLP-1 receptor probe/cyclophilin control probe (Fig. 1), and a 30% decrease was still evident after 24 h of treatment (Fig. 4) (0.49 ± 0.04 vs. 0.71 ± 0.06 arbitrary units in controls, $P < 0.05$).

4. Discussion

Our results demonstrate for the first time the presence of GLP-1 receptor mRNA in cells (C cells) which synthesizes and produces CT and CGRP peptides by alternative splicing of the primary transcript of the CT gene. The specificity of the amplified products was directly established by hybridization with a rat GLP-1 receptor-specific probe. The messenger expressed in CA-77 cells is polyadenylated as we have used oligo dT and reverse transcriptase to obtain the first cDNA copy. The presence of GLP-1 receptor mRNA was substantiated by the positive signal observed with Northern hybridization. As in a rat insulinoma cell line (INS-1) [9], two mRNAs of 2.7 and 3.6 kb could be detected in the rat CA-77 C cell line. GLP-1 receptor mRNA expression has been found to be highly expressed in both rat and human pancreatic islets [19,20]. We found that GLP-1 (7–37) induced a down-regulation of GLP-1 receptor mRNA in CA-77 C cells. In cultured rat pancreatic islets, it was recently reported that GLP-1 receptor mRNA levels remained unchanged under all conditions that altered intracellular cAMP levels [21]. Our observation suggested that regulation of GLP-1 receptor mRNA could be different in CA-77 C cells. Glucocorticoids were found to change slightly the level of GLP-1 receptor mRNA in CA-77 cells, since dexamethasone, after a transient increase, down-regulated GLP-1 receptor mRNA levels by approx. 30%. Our data are in agreement with those in cultured rat

pancreatic islets where dexamethasone decreased by 50% after 48 h the GLP-1 receptor mRNA level [21].

The presence of a functional GLP-1 receptor in CA-77 C cells was also ascertained by the use of specific agonists and antagonists of such a receptor.

As in the Syrian hamster insulinoma [18], the cAMP production in CA-77 C cells was similarly increased by GLP-1 (7–37), and the agonists exendin 3 and exendin 4. Moreover, this cAMP induction was blunted by exendin (9–39) a specific antagonist of GLP-1 receptor [18], and the potency of GLP-1 (7–37) was > glucagon > oxyntomodulin. All these results indicate the presence of GLP-1 receptors in CA-77 C cells coupled to adenylyl cyclase.

Our results also showed that GLP-1 (7–37) stimulated the secretion of both CGRP and CT in C cells since the release in the culture medium of both peptides was increased 1 h after addition of the hormone. The cellular content of CGRP was 20% decreased 3 h after continuous exposure to GLP-1 (7–37) maybe as a consequence of the elevated secretion. The changes in CGRP and CT secretion were associated with altered levels of CGRP and CT mRNAs. The steady-state level of CGRP and CT mRNAs was increased in face of a drop in the cell content of both peptides. This mechanism may be viewed as an adaptation to the elevated secretion which reduces the cellular content of CGRP and CT. If cAMP and phorbol esters are known to increase CT gene expression [22,23], GLP-1 (7–37) is the first hormonal peptide reported to increase both CGRP and CT mRNAs. At this time, GLP-1 (7–37) is only known as a potent stimulator of insulin secretion in the presence of glucose [6], this effect being mediated by activation of adenylate cyclase and a rise in the intracellular concentration of cAMP [24]. The secretion of GLP-1 is stimulated by carbohydrate intake, the absorption of glucose through the sodium/glucose cotransporter stimulates the secretion of GLP-1 [25]. Our data suggest that GLP-1 could be one of the intestinal molecules controlling CT secretion.

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