

# Glucagon-like peptide-1 (GLP-1) and glucose metabolism in human myocytes

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## Abstract

Glucagon-like peptide-1 (GLP-1) has been shown to have insulin-like effects upon the metabolism of glucose in rat liver, muscle and fat, and on that of lipids in rat and human adipocytes. These actions seem to be exerted through specific receptors which, unlike that of the pancreas, are not – at least in liver and muscle – cAMP-associated. Here we have investigated the effect, its characteristics, and possible second messengers of GLP-1 on the glucose metabolism of human skeletal muscle, in tissue strips and primary cultured myocytes. In muscle strips, GLP-1, like insulin, stimulated glycogen synthesis, glycogen synthase *a* activity, and glucose oxidation and utilization, and inhibited glycogen phosphorylase *a* activity, all of this at physiological concentrations of the peptide. In cultured myotubes, GLP-1 exerted, from  $10^{-13}$  mol/l, a dose-related increase of the D-[U- $^{14}$ C]glucose incorporation into glycogen, with the same potency as insulin, together with an activation of glycogen synthase *a*; the effect of  $10^{-11}$  mol/l GLP-1 on both parameters was additive to that induced by the equimolar amount of insulin. Synthase

*a* was still activated in cells after 2 days of exposure to GLP-1, as compared with myotubes maintained in the absence of peptide. In human muscle cells, exendin-4 and its truncated form 9–39 amide (Ex-9) are both agonists of the GLP-1 effect on glycogen synthesis and synthase *a* activity; but while neither GLP-1 nor exendin-4 affected the cellular cAMP content after 5-min incubation in the absence of 3-isobutyl-1-methylxanthine (IBMX), an increase was detected with Ex-9. GLP-1, exendin-4, Ex-9 and insulin all induced the prompt hydrolysis of glycosylphosphatidylinositols (GPIs). This work shows a potent stimulatory effect of GLP-1 on the glucose metabolism of human skeletal muscle, and supports the long-term therapeutic value of the peptide. Further evidence for a GLP-1 receptor in this tissue, different from that of the pancreas, is also illustrated, suggesting a role for an inositolphosphoglycan (IPG) as at least one of the possible second messengers of the GLP-1 action in human muscle.

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## Introduction

Glucagon-like peptide-1 (GLP-1) is an incretin hormone which helps to regulate plasma glucose levels by not only enhancing pancreatic  $\beta$ -cell secretion (Montrose-Rafizadeh *et al.* 1994) and diminishing blood glucagon and somatostatin concentrations (Gutniak *et al.* 1992), but also by acting independently of the circulating insulin levels (Gutniak *et al.* 1992, D'Alessio *et al.* 1994, 1995). To improve glucose utilization, GLP-1 may have peripheral activity in insulin-sensitive tissues; in fact, some insulin-like effects of GLP-1 on glucose metabolism in liver and skeletal muscle have been shown by *in vitro* studies in normal (Valverde *et al.* 1994, Villanueva-Peñacarrillo *et al.* 1994) and diabetic (Morales *et al.* 1997) rats, such as the stimulation of glycogen synthesis, glycogen synthase *a* activity, and glucose oxidation and utilization.

In the adipose tissue of the rat, it was observed that GLP-1 not only exerts an action on lipid metabolism – dose-dependently lipolytic and/or lipogenic (Ruiz-Gande *et al.* 1992, Perea *et al.* 1997) – but also, as in liver and muscle, that it stimulates parameters involved in glucose metabolism (Perea *et al.* 1997).

In liver (Villanueva-Peñacarrillo *et al.* 1995a), muscle (Delgado *et al.* 1995, Villanueva-Peñacarrillo *et al.* 1995b, Yang *et al.* 1998) and fat (Mérida *et al.* 1993, Valverde *et al.* 1993), GLP-1 seems to act through specific receptors, apparently different – at least in liver and muscle – in structure or signaling pathway from that in the pancreas, which is cAMP-associated (Thorens 1992). An inositolphosphoglycan (IPG) has already been proposed as one of the possible second messengers in the GLP-1 effect in these tissues, by studies in BC<sub>3</sub>H myocytes (Galera *et al.* 1996), HEP hepatoma cells (Trapote *et al.* 1996) and rat hepatocytes and adipocytes (Márquez *et al.* 1998).

In this work, we have studied the effects, characteristics, and possible mechanisms of action, of GLP-1 upon muscle glucose metabolism in man.

## Materials and Methods

### Chemicals

Human GLP-1 (7–36)amide (GLP-1, Bachem AG, Bubendorf, Switzerland); pork insulin (Novo Biolabs, Bagsvaerd, Denmark); exendin 1–39 amide (Ex-4) and exendin 9–39 amide (Ex-9) were gifts from Dr John Eng (VAMC, New York, NY, USA); HAM's F-10, fetal bovine serum (FBS) and  $\alpha$ -MEM ( $\alpha$ -modified Eagle medium Biochrom KG, Berlin, Germany); dexamethasone, bovine serum albumin, fraction V (BSA), penicillin–streptomycin, trypsin, EDTA, collagen from rat tail and glutamine (Sigma Chemical Co., St Louis, MO, USA); D-[U-<sup>14</sup>C]glucose and D-[5-<sup>3</sup>H]glucose (Amersham, Chalfont, Bucks, UK); trichloroacetic acid (TCA, Merck, Darmstadt, Germany); human epidermal growth factor (hEGF) and amphotericin B (Clonetics, Palo Alto, CA, USA); D-glucose anhydrous (Scharlau, Barcelona, Spain); HCl (Panreac, Barcelona, Spain); *myo*-[1-<sup>3</sup>H]inositol and EN<sup>3</sup>HANCE (Du Pont NEN, Boston, MA, USA); skeletal muscle cell basal medium (SKBM, Promocell, Heidelberg, Germany); fetuin FBS (Calbiochem-Novabiochem, Darmstadt, Germany); gentamicin (Lab. Normon, S.A., Madrid, Spain); hyamine hydroxide (Hopkins and Williams, Chendewell Health, Essex, UK); <sup>3</sup>HOH and cAMP[<sup>125</sup>I]RIA kits (NEN Du Pont Co., Brussels, Belgium); Ultima Gold scintillation liquid (Packard, Gröningen, The Netherlands).

### Biological material

Human *vastus lateralis* and *cremaster* muscle pieces were obtained from normal-weight subjects (BMI <26 kg/m<sup>2</sup>) undergoing Richard's and inguinal hernia surgery respectively. The consent was obtained from the subjects after the nature of the procedure was explained, and the study was approved by the Ethic Committee of the Fundación Jiménez Díaz, Madrid, in accordance with the guidelines proposed in The Declaration of Helsinki.

### Cell culture

After removal, *vastus lateralis* tissue ( $\approx$  100 mg) was immediately transported to the laboratory in HAM's F-10. The isolation of myoblasts was performed as described previously with minor modifications (Blau & Webster 1981, Henry *et al.* 1995). Muscle was cleaned of blood with HAM's F-10, dissected from visible connecting tissue, and then subjected to digestion, in sterile conditions, by three consecutive treatments of 20 min,

each with trypsin–EDTA (0.05%–0.02%, w/v), at room temperature; after every treatment, dispersed cells were collected in HAM's F-10 and kept at 4 °C. When tissue dispersion was completed, 10% FBS (v/v) was added, and cells were then sedimented at 900 g for 4 min and finally resuspended in 5 ml SKGM growing medium (SKBM with added 10 ng/ml hEGF, 0.5 mg/ml fetuin FBS, 0.39  $\mu$ g/ml dexamethasone, 0.5 mg/ml BSA, 50  $\mu$ g/ml gentamicin, 50 ng/ml amphotericin B, 50 UI/ml–50  $\mu$ g/ml penicillin–streptomycin, 2% FBS). To reduce the number of fibroblasts, dispersed tissue was placed in a 100 mm diameter collagen-free well and kept for 20 min at 37 °C. The non-attached cells were seeded in a rat collagen-treated 75 cm<sup>2</sup> flask (Costar Co., Cambridge, MA, USA), and cultured at 37 °C in SKGM growing medium until reaching  $\approx$ 80% confluence (4–6 weeks), the medium being exchanged every 3 days. After gentle trypsinization (0.025% trypsin–0.01% EDTA, w/v), cells were quantified and newly seeded in six-well culture slides at a density of 20 000 cells/well, and further cultured until 70–80% confluence (4–6 weeks); then the growing medium was substituted by that for fusion, consisting of  $\alpha$ -MEM with added 2% FBS, 1% penicillin–streptomycin (v/v) and 2% glutamine, and cells were further cultured until differentiation into myotubes occurred (3–4 days).

Before and after the differentiation process, part of the wells from some of the cell cultures were kept at –70 °C for determination of changes in creatinine kinase activity (Gerhardt 1983), which represented an increase from  $43 \pm 9$  U/g protein to  $116 \pm 15$  U/g ( $n=6$  from three subjects) after fusion.

### Glycogen synthase a and glycogen phosphorylase a activities

The methods used were those already described in detail for rat hepatocytes (Valverde *et al.* 1994) and skeletal muscle (Villanueva-Peñacarrillo *et al.* 1994). *Cremaster* muscle strips (four to six per each muscle), after 30-min pre-incubation at 37 °C in Krebs–Ringer bicarbonate (KRB) buffer containing 1% BSA and 5 mmol/l D-glucose, were incubated for 10 min at 37 °C in the same medium as above, and in the absence and presence of peptides; always, at least one of the muscle strips of each subject was incubated in the absence of peptides for paired control. Primary culture cells from each subject were incubated for 10 min in 1 ml  $\alpha$ -MEM, in the absence (for paired control) or presence of peptides. The cells or tissue samples were immediately homogenized and frozen until the enzymatic activities were assayed as by Hue *et al.* (1975), except for the final glycogen extraction, which was done according to Fleig *et al.* (1984).

### Glycogen synthesis, <sup>14</sup>CO<sub>2</sub>, <sup>3</sup>HOH and lactate production

D-[U-<sup>14</sup>C]glucose incorporated into glycogen, <sup>14</sup>CO<sub>2</sub> and <sup>3</sup>HOH release, and lactate production, were measured

simultaneously in the same *cremaster* muscle experimental samples.

*Cremaster* muscle strips, after 30-min pre-incubation at 37 °C in 1.5 ml KRB containing 1% BSA and 5 mmol/l D-glucose, were incubated for 60 min in the same medium with 0.35 µCi D-[U-<sup>14</sup>C]glucose and 0.3 µCi D-[5-<sup>3</sup>H]glucose, and in the presence of peptides, as described previously (Morales *et al.* 1997); at least one of the strips from each subject, serving as paired control, was incubated in the absence of peptide. Primary culture myocytes from each subject were incubated for 60 min at 37 °C in 250 µl α-MEM, containing 1.5 µCi D-[U-<sup>14</sup>C]glucose, and in the absence (for paired control) and presence of peptides. In both tissue and cells, the D-[U-<sup>14</sup>C]glucose incorporated into glycogen was extracted and determined according to the procedure described by Cuendet *et al.* (1976).

To measure <sup>14</sup>CO<sub>2</sub> and <sup>3</sup>HOH in *cremaster* muscle strips, the basic procedure described in detail elsewhere (Cámara *et al.* 1991) was followed. In brief, two cups with cellulose Whatman paper, one containing 0.5 ml 0.1 mol/l HCl for <sup>3</sup>HOH uptake, were hung from the rubber stopper vial during the incubation period. Then, 250 µl hyamine hydroxide were injected into the second cup, for <sup>14</sup>CO<sub>2</sub> uptake; after 3 min at 4 °C, the muscle tissue was removed, and the vials with the cups were maintained at 37 °C for 30 min followed by 60 min at 25 °C; then the cups were placed separately in 5 ml of scintillation liquid for <sup>3</sup>H and <sup>14</sup>C content measurements. To control the <sup>3</sup>HOH recovery, 1.5 ml of the same medium containing 4 nCi <sup>3</sup>HOH (NEN Du Pont Co., Brussels, Belgium), without tissue, underwent the whole procedure in each experimental set. Double-channel counting indicated the absence of cross-contamination. The lactate content in the incubation medium was enzymatically assayed (Hohorst 1965).

#### cAMP

Primary culture myocytes were incubated for 5 and 30 min at 37 °C in 500 µl α-MEM, in the absence of 3-isobutyl-methylxanthine (IBMX), and in the absence (control) and presence of peptides, following the procedure already described for rat hepatocytes (Valverde *et al.* 1994).

#### IPGs

IPGs were measured indirectly (Saltiel *et al.* 1986) as the hydrolysis of glycosylphosphatidylinositols (GPIs). Primary culture myocytes (2 × 10<sup>4</sup> cells per well) from each subject were pre-labeled, for 60 min at 37 °C, with 0.2 µCi myo-[<sup>3</sup>H]inositol in 0.75 ml α-MEM with added 0.1% BSA, 1% glutamine and 1% antibiotics; the radioactive medium was then removed and cells were incubated in fresh α-MEM for different time periods (0.5–10 min) in the absence (control) and presence of peptides. Incubation

was interrupted by addition of 10% TCA at 4 °C, and the radioactive GPI content was extracted and determined in the respective precipitates, as previously described in detail (Márquez *et al.* 1998).

#### Statistical study

Results are expressed as means ± S.E.M., together with the number of observations. The statistical significance ( $P < 0.05$ ) of the increments was assessed by the Student's *t*-test and, in some cases, by one-way ANOVA and, when statistically significant ( $P < 0.05$ ), it was followed by the least significant differences (LSD) test for *post hoc* multiple comparisons, using the SPSS statistical software.

## Results

### Glucose metabolism

Table 1 shows the respective control value of glycogen synthesis, glycogen synthase *a* and phosphorylase *a* activities, and glucose oxidation and utilization, in human *cremaster* muscle strips, as well as the effect of GLP-1 and insulin on these parameters, expressed as percentage of the paired control obtained in the absence of peptide. As already reported in rat skeletal muscle (Villanueva-Peñacarrillo *et al.* 1994), GLP-1 significantly increased the D-[U-<sup>14</sup>C]glucose incorporation into glycogen at 10<sup>-10</sup> mol/l, as did 10<sup>-9</sup> mol/l insulin; the rise in glycogen synthesis by GLP-1 was accompanied by an increase in glycogen synthase *a* and a reduction in glycogen phosphorylase *a* activities, and by a stimulation of glucose oxidation (<sup>14</sup>CO<sub>2</sub> produced from D-[U-<sup>14</sup>C]glucose) and utilization (tritiated water from D-[5-<sup>3</sup>H]glucose, and lactate release), all this to a magnitude equivalent to the effect induced by insulin.

Figure 1A represents the effect of GLP-1 and that of insulin on the incorporation of D-[U-<sup>14</sup>C]glucose into glycogen in human myotubes, expressed as percentage of the paired control value obtained within the same cell preparation in the absence of peptide. GLP-1 caused a concentration-related stimulation of the glycogen synthesis from 10<sup>-13</sup> mol/l of the peptide, which was already significant, and maximal, at 10<sup>-10</sup> mol/l, and also statistically significant thereafter up to 10<sup>-7</sup> mol/l GLP-1. Unlike in previous observations in rat skeletal muscle (Villanueva-Peñacarrillo *et al.* 1994), the potency of GLP-1 at exerting glycogenic action in human muscle cells was, qualitatively and quantitatively, apparently indistinguishable from that of insulin; however, in this group of experiments, the cells showed a trend toward a modest desensitization in response to GLP-1 at 10<sup>-7</sup> mol/l. In cells from one subject, the magnitude of the effect on glycogen synthesis of 10<sup>-10</sup> mol/l GLP-1 and 10<sup>-10</sup> mol/l insulin, tested in combination (Fig. 1B), was

**Table 1** Effect of glucagon-like peptide-1 (GLP-1) and insulin on glycogen synthesis and glucose metabolism in strips of normal human skeletal muscle (mean  $\pm$  S.E.M. ( $n$ =number of observations from 16 subjects))

	Control No peptide	% of paired control		
		GLP-1		Insulin 10 <sup>-9</sup> mol/l
		10 <sup>-10</sup> mol/l	10 <sup>-9</sup> mol/l	
G-synthesis	5.7 $\pm$ 3.3 (9) nmol/mg protein	129 $\pm$ 5 (7)	111 $\pm$ 7 (8)	123 $\pm$ 9 (7)
One-way ANOVA, $P=0.011$	LSD test, vs control	$P=0.002$	$P=0.205$	$P=0.013$
G-synthase a	1.3 $\pm$ 0.3 (22) U/g protein	135 $\pm$ 22 (7)	147 $\pm$ 14 (17)	148 $\pm$ 16 (16)
One-way ANOVA, $P=0.030$	LSD test, vs control	$P=0.137$	$P=0.010$	$P=0.010$
G-phosphorylase a	206 $\pm$ 30 (36) U/g protein	76 $\pm$ 13 (12)	86 $\pm$ 5 (23)	83 $\pm$ 6 (30)
One-way ANOVA, $P=0.028$	LSD test, vs control	$P=0.010$	$P=0.067$	$P=0.016$
<sup>14</sup> CO <sub>2</sub>	143 $\pm$ 24 (14) pmol/mg protein	168 $\pm$ 14 (6)	—	134 $\pm$ 12 (7)
One-way ANOVA, $P=0.001$	LSD test, vs control	$P<0.0001$		$P=0.024$
<sup>3</sup> HOH	51 $\pm$ 8 (19) nmol/mg protein	134 $\pm$ 17 (10)	148 $\pm$ 11 (8)	131 $\pm$ 10 (14)
One-way ANOVA, $P=0.016$	LSD test, vs control	$P=0.024$	$P=0.004$	$P=0.024$
Lactate	68 $\pm$ 11 (10) $\mu$ mol/g protein	125 $\pm$ 11 (9)	—	134 $\pm$ 13 (9)
One-way ANOVA, $P=0.032$	LSD test, vs control	$P=0.061$		$P=0.013$

indistinguishable from the respective stimulated values obtained by each peptide individually; however, at 10<sup>-11</sup> mol/l, this combination induced an increment of the control which was almost the sum of those exerted by each individual hormone.

GLP-1 also induced a small but highly significant increment ( $P<0.001$  at 10<sup>-10</sup> and 10<sup>-9</sup> mol/l) in the glycogen synthase *a* activity paired control value (28.0  $\pm$  3.3 mU/g protein,  $n=57$  from nine subjects). Although this effect of the peptide when tested at 10<sup>-10</sup> mol/l was equal in magnitude to that induced by the equimolar amount of insulin, at 10<sup>-9</sup> mol/l (54  $\pm$  9%  $\Delta$  of control,  $n=29$ ,  $P<0.01$ ) it was shown to be significantly higher ( $P<0.01$  vs 10<sup>-9</sup> mol/l insulin: 22  $\pm$  5%  $\Delta$ ,  $n=9$ ,  $P<0.01$  vs control). When GLP-1 and insulin were tested in combination in myocytes from one subject (Fig. 1C), both at 10<sup>-10</sup> mol/l, the value obtained was not different from those produced by each individual hormone; yet, the increment induced by the combination of both at 10<sup>-11</sup> mol/l represented the sum of those exerted by each hormone.

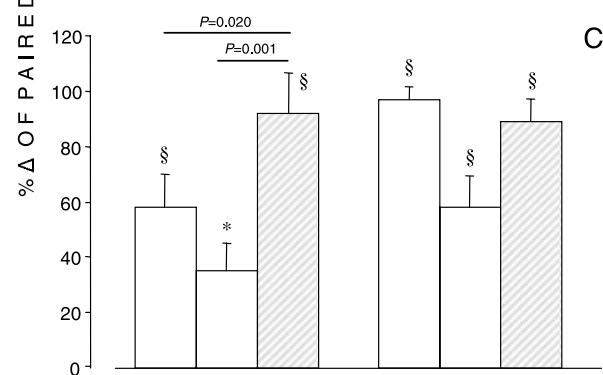
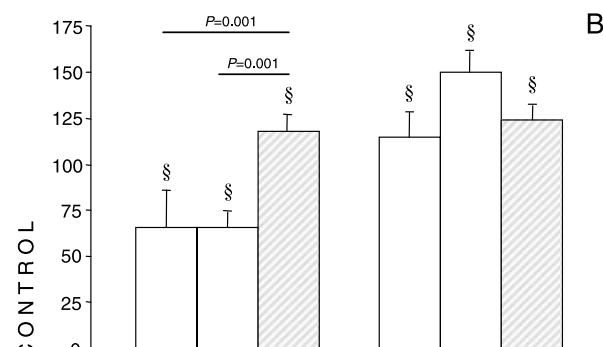
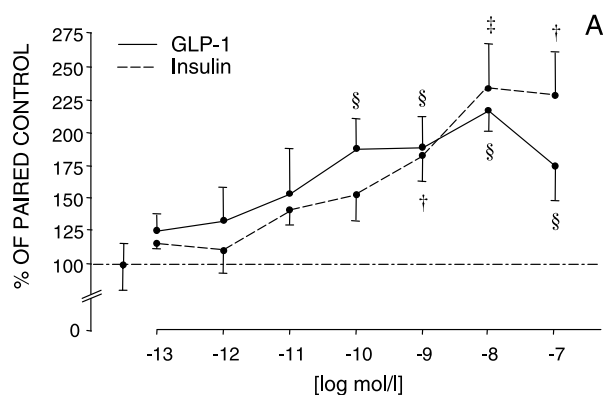
#### Prolonged GLP-1 action

To learn about the effect of a prolonged action of GLP-1 upon glycogen metabolism in the skeletal muscle, we measured the glycogen synthesis and glycogen synthase *a* activity in primary culture myocytes from two subjects, after 48 h of culture in  $\alpha$ -MEM in the absence and presence of added 10<sup>-7</sup> mol/l GLP-1 or insulin. To correct the loss of stability of the hormones during the experimental conditions, mainly that of GLP-1 (previously estimated at 35.6  $\pm$  2.1% radioactive 10<sup>-7</sup> mol/l GLP-1 degradation after 10 h in the presence of cells,  $n=6$ , by measuring the 10% TCA-precipitable peptide), the medium without or with GLP-1 or insulin was replaced

by a fresh one every 8 h. As a result, we observed that after a 2-day treatment with GLP-1, the D-[U-<sup>14</sup>C]glucose incorporated into glycogen was 41  $\pm$  8% higher ( $n=13$ ,  $P<0.001$ ) with respect to the control value obtained in cells maintained in culture without peptide (14.4  $\pm$  0.5 nmol glucose/mg protein,  $n=12$ ); this increment was much lower ( $P<0.001$ ) though than that detected after 2 days in the presence of insulin (137  $\pm$  10%  $\Delta$  of control,  $n=15$ ,  $P<0.001$ ). Also, after either GLP-1 or insulin treatment, an increase of the control glycogen synthase *a* activity (78  $\pm$  4 mU/g protein,  $n=9$ ), although very modest, was observed (17  $\pm$  6%  $\Delta$  of control,  $n=20$ , and 19  $\pm$  7%  $\Delta$ ,  $n=16$ , GLP-1 and insulin respectively, both  $P<0.02$ ).

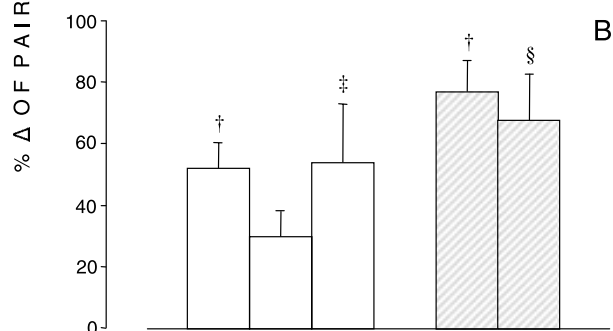
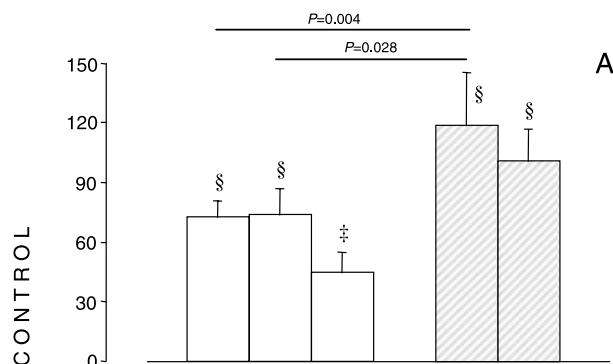
#### Characterization of GLP-1 receptor

In an approach to characterize the GLP-1 effect on human muscle glucose metabolism, we tested in primary culture myocytes the action of Ex-4, and that of its truncated form, Ex-9 – agonist and antagonist, respectively, of the GLP-1 receptors in several cell systems – upon glycogen synthesis and synthase *a* activity, and also their interaction with GLP-1. Ex-4, at 10<sup>-10</sup> mol/l (Fig. 2A), induced an increment (74  $\pm$  13%  $\Delta$  of paired control,  $n=21$  from four subjects) of the glycogen synthesis, equivalent to that exerted by the equimolar amount of GLP-1 (73  $\pm$  8%  $\Delta$ ,  $n=73$  from ten subjects). Surprisingly, in these cells, 10<sup>-10</sup> mol/l Ex-9 also increased glycogen synthesis, to a magnitude (45  $\pm$  10%  $\Delta$ ,  $n=34$  from six subjects) equal to that exerted by either GLP-1 or Ex-4. In previous studies using the same exendin batches as in this work, we have documented that Ex-9, when intravenously infused in rats, inhibits the insulinotropic effect of GLP-1 (Cancelas *et al.* 2001a), whereas Ex-4 stimulates insulin secretion (Cancelas *et al.* 2001b). When tested together, the effect of



GLP-1	+	-	+	+	-	+
Insulin	-	+	+	-	+	+
	10 <sup>-11</sup> mol/l			10 <sup>-10</sup> mol/l		

**Figure 1** Effect of glucagon-like peptide-1 (GLP-1) and insulin on glycogen synthesis and glycogen synthase *a* activity in primary cultured myocytes. (A) Dose-response of glycogen synthesis ( $n=3-15$ ) in cells from four subjects; (B) combined effect of GLP-1 and insulin on glycogen synthesis ( $n=8-18$ ), and (C) glycogen synthase *a* activity ( $n=3-6$ ). Values (mean  $\pm$  s.e.m.) are relative to the respective paired control obtained in the absence of peptide (A:  $6.8 \pm 1.2$  nmol/mg protein,  $n=19$  from four subjects; B:  $4.9 \pm 0.3$  nmol/mg protein,  $n=6$  from one subject; C:  $29.0 \pm 2.6$  mU/g protein,  $n=6$  from one subject). One-way ANOVA: (A) GLP-1,  $P=0.001$ ; insulin,  $P<0.0001$ ; (B)  $P<0.0001$ ; (C)  $P<0.0001$ . LSD test: vs control, \* $P<0.05$ , † $P<0.01$ , ‡ $P<0.001$ , § $P<0.0001$ ; significance between increments are indicated on the Figure.



GLP-1 10 <sup>-10</sup> mol/l	+	-	-	+	+
Ex-4 10 <sup>-10</sup> mol/l	-	+	-	+	-
Ex-9 10 <sup>-10</sup> mol/l	-	-	+	-	+

**Figure 2** Effect of exendin (Ex)-4 and Ex-9, alone and in combination with GLP-1, on (A) glycogen synthesis in primary cultured myocytes ( $n=21-73$ ) from 12 subjects and (B) glycogen synthase *a* activity ( $n=4-15$ ) from six subjects. Values (mean  $\pm$  s.e.m.) are relative to the respective paired control obtained in the absence of peptide (A:  $8.8 \pm 0.9$  nmol/mg protein,  $n=64$  from 12 subjects; B:  $41 \pm 3$  mU/g protein,  $n=42$  from six subjects). One-way ANOVA: (A)  $P<0.0001$ ; (B),  $P=0.001$ . LSD test vs control: † $P<0.01$ , ‡ $P<0.001$ , § $P<0.0001$ ; significance between increments are indicated on the figure.

GLP-1 combined with Ex-4 ( $119 \pm 27\%$   $\Delta$ ,  $n=24$  from six subjects) or Ex-9 ( $101 \pm 16\%$   $\Delta$ ,  $n=40$  from six subjects), was higher than either individual value. The same characteristics of the action of both exendins in glycogen synthesis were detected upon glycogen synthase *a* activity (Fig. 2B), the Ex-9 stimulating effect ( $54 \pm 19\%$   $\Delta$  of paired control,  $n=16$  from three subjects) being similar to that induced by either GLP-1 ( $52 \pm 8\%$   $\Delta$ ,  $n=15$  from three subjects) or Ex-4 ( $30 \pm 8\%$   $\Delta$ ,  $n=8$  from four subjects); in this case also, the effects of GLP-1 combined with Ex-4 ( $77 \pm 10\%$   $\Delta$ ,  $n=4$  from three subjects) or with Ex-9 ( $68 \pm 14\%$   $\Delta$ ,  $n=10$  from three subjects) were both higher than the respective individual ones, as observed in glycogen synthesis, but the differences did not achieve statistical significance.

**Table 2** Effect of  $10^{-8}$  mol/l GLP-1, Ex-4 or Ex-9 on the cAMP content in primary culture human myotubes (mean  $\pm$  S.E.M. ( $n$ =number of observations from one subject)). One-way ANOVA,  $P<0.0001$ 

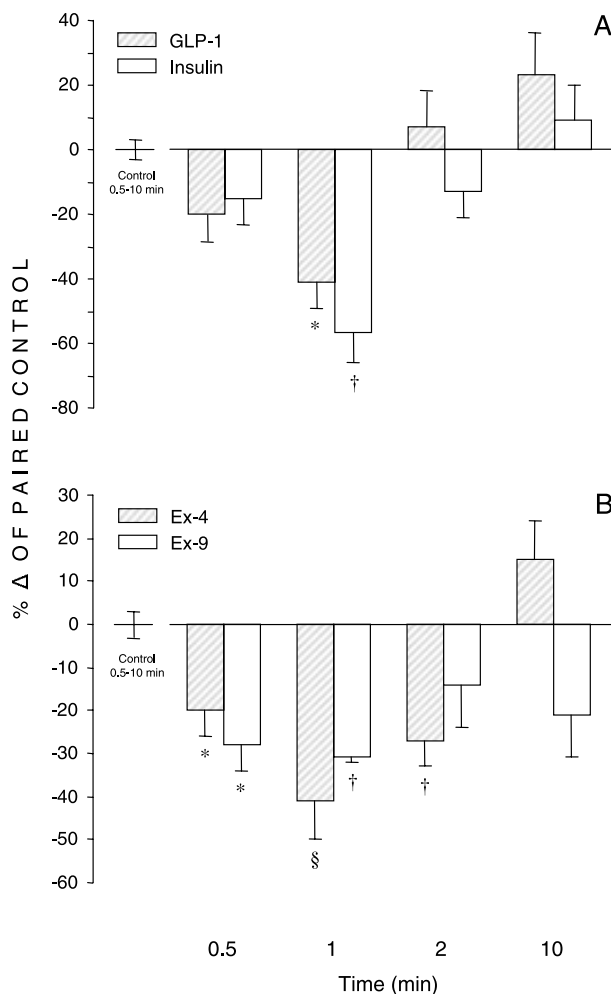
	5 min		30 min	
	Control	% of paired control	Control	% of paired control
GLP-1	$42 \pm 2$ pmol/mg protein (9)	$114 \pm 7$ (11)	$77 \pm 6$ pmol/mg protein (10)	$60 \pm 6$ (12)
	LSD test, vs control	$P=0.068$	LSD test, vs control	$P<0.0001$
Ex-4	$84 \pm 5$ (6)	$84 \pm 5$ (6)	$84 \pm 4$ (6)	$84 \pm 4$ (6)
	LSD test, vs control	$P=0.040$	LSD test, vs control	$P=0.024$
Ex-9	$131 \pm 6$ (5)	$131 \pm 6$ (5)	$61 \pm 5$ (6)	$61 \pm 5$ (6)
	LSD test, vs control	$P=0.001$	LSD test, vs control	$P<0.0001$

To search for possible second messengers in the GLP-1 action in human muscle glucose metabolism, and also in that of exendins, we measured the cellular cAMP content in primary culture myocytes from one subject, incubated in the absence and presence of GLP-1, Ex-4 or Ex-9, for 5 or 30 min, and also their effect, and that of insulin, on IPG generation from the hydrolysis of GPIs, in cell samples from three subjects. We observed (Table 2) no changes in the cellular cAMP control content after 5 min in the presence of  $10^{-8}$  mol/l GLP-1, but a clear decrease of the respective control value was demonstrated after a 30-min incubation period; with the equimolar amount of Ex-4, a statistically significant reduction, although small, of the cAMP content was already detected at 5 min of incubation and was still observed at 30 min. Unlike Ex-4, Ex-9 exhibited a clear increase in the cellular cAMP content after 5 min of incubation, which fell below the control value at 30 min.

For cellular GPI content, as the values obtained in primary culture myocytes from three subjects incubated in the absence of added peptides from 0.5 to 10 min did not show statistical difference at any time point, the mean of these values was used as control. GLP-1, at  $10^{-9}$  mol/l, induced a rapid decrease of the radioactive GPIs (Fig. 3A), already detected in the first 30 s in the presence of the peptide, and statistically significant at 1 min; this reduction was followed by a progressive recovery of the control level, with a maximal apparent value at 10 min. The dynamic in GPI content, induced by GLP-1, was similar to that observed with the equimolar amount of insulin. Ex-4 and Ex-9 at  $10^{-9}$  mol/l both exerted, like GLP-1, a rapid reducing effect on myotube GPI content, observed at 0.5-, 1- and 2-min incubation in the presence of either peptide, which was followed by a recovery of the control value at 10 min.

## Discussion

The present data document that GLP-1 exerts insulin-like effects upon glucose metabolism in human skeletal



**Figure 3** Effect of  $10^{-9}$  mol/l GLP-1 or insulin (A), and that of Ex-4 or Ex-9 (B) on the hydrolysis of glycosylphosphatidylinositols (GPIs) in primary cultured myocytes. Values (mean  $\pm$  S.E.M.,  $n=3$  from three subjects) are relative to the respective paired control obtained in cells incubated in the absence of peptide ( $2008 \pm 55$  d.p.m. per 20 000 cells,  $n=40$  from three subjects). One-way ANOVA: (A) GLP-1,  $P=0.035$ ; insulin,  $P=0.002$ ; (B) Ex-4,  $P<0.0001$ ; Ex-9,  $P=0.050$ . LSD test vs control: \* $P<0.05$ , † $P<0.01$ , § $P<0.0001$ .

muscle. The GLP-1 dose–response of glycogen synthesis was similar to that of insulin, with an  $ED_{50}$  of about  $10^{-11}$  mol/l. At this submaximal concentration, the combined effect of both peptides on both glycogen synthesis and glycogen synthase *a* activity was additive, whereas at  $10^{-10}$  mol/l no further increment was detected, indicating that GLP-1 may share the insulin-signaling pathway at some post-receptor level. These *in vitro* effects were previously observed in skeletal muscle from normal (Villanueva-Peñacarrillo *et al.* 1994) and diabetic (Morales *et al.* 1997) rats, mice (O'Harte *et al.* 1997), and also in the rat myocyte cell line L6 (Yang *et al.* 1998), rat adipocytes (Oben *et al.* 1991, Miki *et al.* 1996, Perea *et al.* 1997) and mice adipocyte cell line 3T3-L1 (Egan *et al.* 1994). The activating action of GLP-1 on glycogen synthesis and synthase *a* activity in human myotubes was maintained after 48 h exposure to the peptide, which supports the long-term therapeutic value of GLP-1.

This action of GLP-1 in muscle is probably being exerted through specific receptors (Delgado *et al.* 1995, Yang *et al.* 1998), with a signaling pathway (Villanueva-Peñacarrillo *et al.* 1994, Delgado *et al.* 1995, Alcántara *et al.* 1997, Yang *et al.* 1998) likely different from the pancreatic one (Thorens 1992). In human myocytes, we have detected either no effect, or a significant decrease in the cellular cAMP content, depending on the time cells had been in the presence of GLP-1 and absence of the phosphodiesterase inhibitor IBMX. This is in accordance not only with our previous observations in rat skeletal muscle (Villanueva-Peñacarrillo *et al.* 1994, Delgado *et al.* 1995, Alcántara *et al.* 1997) but also with those from other investigators in L6 myocytes (Yang *et al.* 1998). The same has been proposed for the GLP-1 receptor in the liver (Valverde *et al.* 1994, Alcántara *et al.* 1997) and in adipose cells (Miki *et al.* 1996, Montrose-Rafizadeh *et al.* 1997), where the peptide activates glycogen synthesis and lipid metabolism respectively, as either no change or a reduction in the cellular cAMP content by GLP-1 has been detected.

Further support for a different kind of GLP-1 receptor in those extrapancreatic tissues is furnished by the present results in human myotubes. In these cells, GLP-1, like insulin, induced the immediate hydrolysis of GPIs – indicating the generation of IPGs – with a global dynamic similar to that previously detected not only in BC3H-1 myocytes (Galera *et al.* 1996) but also in a hepatoma cell line (Trapote *et al.* 1996) and in rat hepatocytes and adipocytes (Márquez *et al.* 1998), suggesting a role for IPGs in the immediate post-receptor mechanism leading to the GLP-1 actions.

Although our previous study in rat skeletal muscle, and in hepatocytes, documented an effect of Ex-9, antagonistic to that of GLP-1 (Alcántara *et al.* 1997), we have found in this work that, in human muscle cells, this peptide acts as an agonist by stimulating glycogen synthesis and glycogen

synthase *a* activity; also, Ex-9 induced – although only within the first minutes of incubation in the absence of IBMX – a small but clear increase in cellular cAMP, in contrast to GLP-1 and Ex-4 by which no change or a reduction was detected. This agonistic action of Ex-9 in human myotubes is in accordance with the work of Yang *et al.* (1998) in L6 myocytes and that of Montrose-Rafizadeh *et al.* (1997) in 3T3-L1 adipocytes, all of which add further evidence for a GLP-1 receptor in the skeletal muscle, different from that in the pancreas.

The presence of these *in vivo* extrapancreatic effects of GLP-1, when intravenously administered, has been suggested in man, normal and diabetic (Gutniak *et al.* 1992, D'Alessio *et al.* 1994, 1995, Shalev *et al.* 1998), and also in depancreatized dogs (Sandhu *et al.* 1999) and diabetic rats (Mizuno *et al.* 1997), but its relevance is questioned by other studies on healthy humans (Toft-Nielson *et al.* 1996, Orskov *et al.* 1996, Larsson *et al.* 1997, Ryan *et al.* 1998), type 2 diabetic patients (Ahren *et al.* 1997, Vella *et al.* 2000) and insulin-dependent diabetic dogs (Freyse *et al.* 1999). Still, a recent work (Vella *et al.* 2001) performed in type 1 diabetic subjects has demonstrated that in the presence of hyperglycemia, hyperinsulinemia and enterally delivered glucose, GLP-1 does not modify splanchnic but increases total body glucose uptake, the skeletal muscle being, most probably, responsible for it. Due to the hormone interactions, in some of the published work it has been difficult to disclose an *in vivo* direct effect of GLP-1 on glucose metabolism; nevertheless, the *in vitro* results, including the present, preclude a direct action of GLP-1 on extrapancreatic tissues participating in the glucose homeostasis.

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