Cell signalling of glucagon-like peptide-1 action in rat skeletal muscle

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Abstract

Glucagon-like peptide-1 (GLP-1), an incretin with glucose-dependent insulinotropic and insulin-independent antidiabetic properties, has insulin-like effects on glucose metabolism in extrapancreatic tissues participating in overall glucose homeostasis. These effects are exerted through specific receptors not associated with cAMP, an inositol phosphoglycan being a possible second messenger. In rat hepatocytes, activation of phosphatidylinositol 3-kinase (PI3K)/protein kinase B (PKB), protein kinase C (PKC) and protein phosphatase 1 (PP-1) has been shown to be involved in the GLP-1-induced stimulation of glycogen synthase. We have investigated the role of enzymes known or suggested to mediate the actions of insulin in the GLP-1-induced increase in glycogen synthase *a* activity in rat skeletal muscle strips.

We first explored the effect of GLP-1, compared with that of insulin, on the activation of PI3K, PKB, p70s6 kinase (p70s6k) and p44/42 mitogen-activated protein kinases (MAPKs) and the action of specific inhibitors of these kinases on the insulin- and GLP-1-induced increment in glycogen synthase *a* activity.

The study showed that GLP-1, like insulin, activated PI3K/PKB, p70s6k and p44/42. Wortmannin (a PI3K inhibitor) reduced the stimulatory action of insulin on glycogen synthase *a* activity and blocked that of GLP-1, rapamycin (a 70s6k inhibitor) did not affect the action of GLP-1 but abolished that of insulin, PD98059 (MAPK inhibitor) was ineffective on insulin but blocked the action of GLP-1, okadaic acid (a PP-2A inhibitor) and tumour necrosis factor-α (a PP-1 inhibitor) were both ineffective on GLP-1 but abolished the action of insulin, and Ro 31–8220 (an inhibitor of some PKC isoforms) reduced the effect of GLP-1 while completely preventing that of insulin.

It was concluded that activation of PI3K/PKB and MAPKs is required for the GLP-1-induced increment in glycogen synthase *a* activity, while PKC, although apparently participating, does not seem to play an essential role; unlike in insulin signaling, p70s6k, PP-1 and PP-2A do not seem to be needed in the action of GLP-1 upon glycogen synthase *a* activity in rat muscle.

Journal of Endocrinology (2004) **180,** 389–398

Introduction

Glucagon-like peptide-1 (GLP-1) is an insulinotropic peptide that has been proposed as a possible tool for the therapy of type 2 diabetes (Gutniak *et al.* 1992); it has incretin character and insulin-independent antidiabetic properties (Gutniak *et al.* 1992, D'Alessio *et al.* 1995), and mimics the effects of insulin on glucose metabolism in the skeletal muscle and liver from normal (Valverde *et al.* 1994, Villanueva-Peñacarrillo *et al.* 1994) and diabetic rats (Morales *et al.* 1997), and also in fat (Perea *et al.* 1997, Villanueva-Peñacarrillo *et al.* 2001*a*). In these extrapancreatic tissues, GLP-1 seems to act through specific receptors (Mérida *et al.* 1993, Valverde *et al.* 1993, Delgado *et al.* 1995, Villanueva-Peñacarrillo *et al.* 1995*a*,*b*, Yang *et al.* 1998) which, unlike the pancreatic one (Thorens 1992), are not associated with an activation of adenylate cyclase (Valverde *et al.* 1994, Villanueva-Peñacarrillo *et al.* 1994, Yang *et al.* 1998). It has been proposed that an inositol phosphoglycan (IPG) could be one of the possible second messengers in the actions of this peptide (Galera *et al.* 1996, Trapote *et al.* 1996, Márquez *et al.* 1998). In fact, a recent report has shown that GLP-1 stimulates glycogen synthase *a* activity and glycogen synthesis in human skeletal muscle strips and primary culture myotubes, as does insulin, and that it fails to modify the cellular cAMP content while stimulating IPG generation (Luque *et al.* 2002). In addition, GLP-1 modulates glucose transport in cultured 3T3-L1 adipocytes (Wang *et al.* 1997), and it seems to control glucose transporter expression at the translational and/or post-translational level in tissues participating in overall glucose homeostasis in normal and

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diabetic rats (Villanueva-Peñacarrillo *et al.* 2001*b*). Insulin-like effects of GLP-1 have additionally been reported in mice abdominal muscle (O'Harte *et al.* 1997) and L6 myotubes (Yang *et al.* 1998), and in rat adipose tissue (Oben *et al.* 1991) and 3T3-L1 adipocytes (Egan *et al.* 1994).

Although some effects of GLP-1 in extrapancreatic tissues are well documented, the nature of its corresponding receptor is as yet unknown. Information about the cellular signalling of the action of GLP-1 has also been, for a long time, very scanty, it being reported only that GLP-1 activates p38 mitogen-activated protein kinase (MAPK), extracellular regulated kinases (ERKs) and phosphatidylinositol 3-kinase (PI3K) in insulinoma cells (Buteau *et al.* 1999, 2001) and in hamster ovary cells transfected with the pancreatic GLP-1 receptor (Montrose-Rafizadeh *et al.* 1999). Very recently, it has been shown that in rat hepatocytes the activation of PI3K/protein kinase B (PKB), protein kinase C (PKC) and protein phosphatase 1 (PP-1), but not PP-2A, seems to mediate the stimulatory action of GLP-1 on glycogen synthase *a*, while MAPKs and p70s6 kinase (p70s6k) could participate in other effects of GLP-1 (Redondo *et al.* 2003).

The entire itinerary and functional consequences of insulin signalling and glucose homeostasis are not available either. However, while a possible participation of p44/42 MAPKs remains unclear (Peak *et al.* 1998), solid data have demonstrated that binding of insulin to its receptor activates PI3K, this enzyme producing phosphatidylinositol trisphosphate (PIP_3), which is considered a second messenger for insulin (Hajduch *et al.* 2001). PIP_3 stimulates the phosphorylation and activation of PKB which has a number of substrates, one being glycogen synthase kinase 3 (GSK3) which is inactivated by phosphorylation (Cross *et al.* 1995). The inhibition of GSK3 could alter the balance between this enzyme and PP-1, leading to dephosphorylation of glycogen synthase and its subsequent activation. But, together with an implication of PKB in the stimulation of glucose uptake and glycogen synthesis by insulin (Ueki *et al.* 1998), it has been shown that direct inhibition of GSK3 in liver cells increases glycogenesis (Armstrong *et al.* 2001); thus, a combined mechanism of both might exist. In addition, some authors have provided evidence for a contribution of a rapamycin-sensitive signalling pathway, involving p70s6k, to the control of glycogen synthesis (Shepherd *et al.* 1995, Hurel *et al.* 1996). From results in human muscle cells, this pathway has been suggested to influence glycogenesis independently of GSK3 (Halse *et al.* 1999). In addition, it has been shown that insulin activates atypical PKC isoforms in a PI3K-dependent manner, and that activation of PKC as well as that of PKB is apparently required for insulin-induced Glut-4 translocation and subsequent stimulation of glucose transport (Litherland *et al.* 2001).

Here we have studied in rat skeletal muscle the effect of GLP-1 on the activation of cellular enzymes known or proposed to mediate insulin actions, and their possible participation in the GLP-1-induced glycogen synthesis.

Materials and Methods

Materials

Human GLP-1 (7–36) amide (GLP-1; Bachem AG, Bubendorf, Switzerland); pork insulin (Novo Biolabs, Bagsvaerd, Denmark); ATP, [γ^{32}P]ATP (30 Ci/nmol), horseradish peroxidase-conjugated donkey anti-rabbit immunoglobulin, Rainbow markers, ECL-Western blotting kit and Hyperfilm ECL (Amersham Pharmacia Biotech, Amersham, Bucks, UK); rapamycin, wortmannin, okadaic acid, phosphatidylinositol and phosphatidylserine (Sigma Aldrich Quimica S.A., Madrid, Spain); tumor necrosis factor-α (TNFα; Clontech Laboratories Inc., Palo Alto, CA, USA); PD98059 and Ro 31–8220 (Calbiochem, La Jolla, CA, USA); rabbit anti-total and anti-phosphorylated form of p44/42 MAP kinase, p70s6k and PKB (Cell Signalling Technology, New England Biolabs, Beverly, MA, USA); rabbit anti-PI3-kinase p85 (Upstate Biotechnology, Lake Placid, NY, USA). All other commonly used chemicals were from Sigma or Merck Pharma Quimica S.A. (Barcelona, Spain). Normal Wistar rats, kept on a standard pellet diet (UAR Panlab, Barcelona, Spain), were obtained from the animal breeding station of the Fundación Jiménez Díaz.

General protocol

Rats (248 ± 4 g, $n=101$) were killed by decapitation, and the soleus muscles were removed and divided into strips (two per muscle) which were individually attached by the tendon to a steel clip. For glycogen synthase *a* activity studies (Morales *et al.* 1997), the strips were preincubated for 60 min at 37 °C in 1·5 ml Krebs–Ringer bicarbonate (KRB) buffer containing 1% bovine serum albumin (BSA) and 5 mM D-glucose, and without or with $2{\cdot}5 \times 10^{-5}$ M PD98059 (a mitogen-activated protein kinase kinase (MEK) inhibitor), 10^{-6} M wortmannin (a PI3K inhibitor), 10^{-7} M rapamycin (a p70S6k inhibitor), 10^{-7} M Ro 31–8220 (a PKC inhibitor), 10^{-6} M okadaic acid (a PP-2A inhibitor) or 5×10^{-8} M TNFα (a PP-1 inhibitor). This was followed by a 10 min incubation at 37 °C in the same medium as above, in the absence and presence of GLP-1 or insulin at 10^{-10} M and 10^{-9} M respectively, as these are the concentrations at which each hormone has previously been shown to exert maximal effect upon muscle glucose metabolism *in vitro* (Villanueva-Peñacarrillo *et al.* 1994, Luque *et al.* 2002). During preincubation and incubation, an atmosphere of O_2/CO_2 (95/5) was maintained in the vial sealed with a rubber stopper. One of the muscle strips from each rat was always incubated in the absence of peptides as a paired control.

The tissue samples were immediately homogenized in a medium containing 100 mM NaF, 35 mM EDTA and 0·5% glycogen (w/v) at pH 7·4, as already described (Villanueva-Peñacarrillo *et al.* 1994), and maintained at −70 °C until the enzymatic activity was assayed.

In another group of experiments, meant to study MAPKs, PI3K, PKB or p70s6k enzyme activities, muscle strips were preincubated for 60 min in KRB with 1% BSA and 5 mM D-glucose, followed by a 3 min incubation in the absence (control, one per rat) or presence of GLP-1 or insulin. Tissue samples were then homogenized at 4 °C in 1·25% Triton X-100 containing 250 mM sucrose, 20 mM Tris/HCl, 2·5 mM $MgCl_2$, 50 mM β-mercaptoethanol, 1·2 mM EGTA, 1 mM Na_3VO_4, 5 mM $Na_4P_2O_7$, 50 mM NaF, 30 U/ml bacitracin, 2 μM leupeptin, 2 μM pepstatin, pH 7·4, and 2 mM phenylmethylsulphonyl fluoride, then maintained at 4 °C for 30 min, and finally centrifuged at 15 000 ***g*** (Arnold *et al.* 1993). The supernatant (tissue lysate), containing cytosol and solubilized membranes, was kept at −70 °C until needed. When dimethyl sulphoxide was required to dissolve some of the tested inhibitors, it was added in the same proportion to all experimental samples.

An aliquot volume was taken from all tissue lysate samples for protein content determination (Bradford 1976). Animal housing and protocols were approved by the Animal Use Committee of the Fundación Jiménez Díaz, Madrid, Spain.

Glycogen synthase a

The frozen tissue homogenates were thawed at 4 °C and their enzymatic activity was assayed, at least in duplicate, as the incorporation of UDP-glucose into glycogen, during 15 min, as previously described in detail (Villanueva-Peñacarrillo *et al.* 1994). In each experimental rat, the mean value of the replicates corresponding to the muscle strip incubated in the absence of peptides or inhibitors was used as the control value.

Immunoblotting

Equal amounts of tissue lysates from each muscle sample were subjected to SDS-PAGE (Laemmli 1970), in parallel with molecular weight markers, on an 8% resolving gel. The separated proteins were then transferred to a nitrocellulose membrane in a semidry system (trans-blot SD semidry transfer cell; BioRad). For immunodetection, a Western blotting kit was used following the manufacturer's instructions, using total and phosphorylated p44/42 MAPK, PKB and p70s6k respective antibodies, and a horseradish peroxidase-conjugated donkey anti-rabbit immunoglobulin second antibody, with detection by the enhanced chemiluminiscence method and quantitation by densitometric scanning of the autoradiography (Villanueva-Peñacarrillo *et al.* 2001*a*). In all experiments, the densitometric value of the band corresponding to the soleus muscle strip incubated in the absence of peptide was used as the control value.

PI3K activity

The enzyme activity was measured directly in p85 immunoprecipitates obtained by treating the muscle lysates with anti-PI3K p85 and subsequent coupling to protein A-agarose (Phung *et al.* 1997). The immunoprecipitates were incubated for 20 min at room temperature with 20 μM [γ^{32}P]ATP (5 μCi/nmol) containing 6·25 mM Hepes, 5 mM $MgCl_2$ and 0·25 mM EGTA, and in the presence of 0·25 mg/ml phosphatidylinositol/phosphatidylserine as substrate. The reaction was interrupted by the addition of 400 μl chloroform/methanol/HCl (1:2:1, by vol), 150 μl chloroform and 150 μl HCl. After centrifugation (10 000 ***g***), the organic phase was treated with an equal volume of methanol/100 mM HCl/2·5 mM EDTA (1:1:1, by volume), and the new organic phase was separated by centrifugation and speed-vac dried. The lipidic extract, redissolved in chloroform, was spotted, together with PIP_3 standard, on a silicagel TLC plate, and developed in *n*-propanol/acetic acid/H_2O (66:2:33, by vol). Plates were dried, and radioactive PIP_3 was subsequently visualized by autoradiography and analyzed by densitometric scanning. In all experiments, the densitometric value of the band corresponding to the muscle strip incubated in the absence of peptide was used as the control value.

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 one-way ANOVA followed by the least significant differences (LSD) test for post-hoc multiple comparisons, using the Statistical Package for the Social Science software.

Results

PI3K/PKB

In rat soleus muscle, GLP-1, at 10^{-10} M, induced a significant increase in the PI3K activity – with respect to the control value obtained in one or two muscle strips from each experimental rat, incubated in the absence of peptide, measured after a 3 min incubation (Fig. 1). As expected, 10^{-9} M insulin also stimulated the PI3K activity, the magnitude of the increment in the generation of PIP_3 being apparently the same as that obtained with 10^{-10} M GLP-1.

In another group of rats, GLP-1, like insulin, also significantly stimulated the phosphorylation of PKB, the

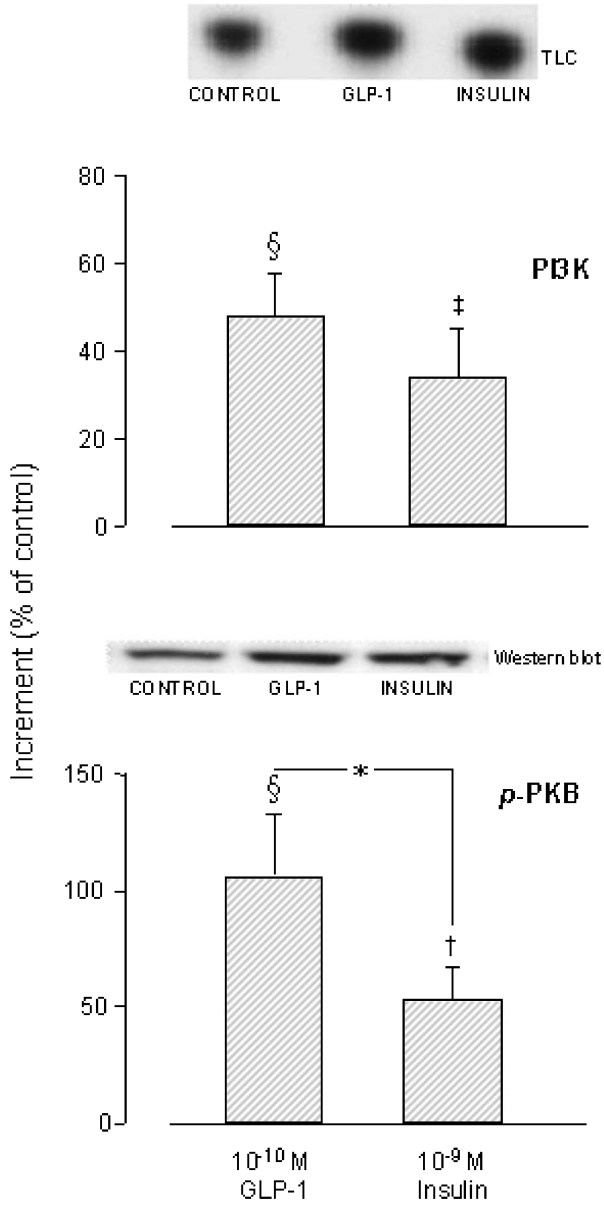


Figure 1 Effect of 10^{-10} M GLP-1 and 10^{-9} M insulin (representative blots and means ± S.E.M., n=4–5 rats) upon PI3K activity and PKB phosphorylation (p), in soleus muscle strips incubated for 3 min. Values are relative to the respective paired control obtained in the muscle sample incubated in the absence of peptide. LSD test: *P=0·024, †P=0·012, ‡P=0·001, §P<0·0001.

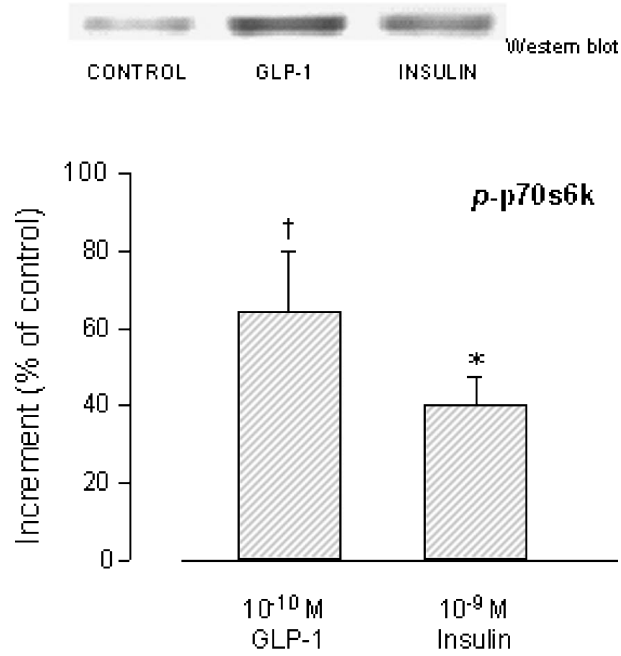


Figure 2 Effect of 10^{-10} M GLP-1 and 10^{-9} M insulin upon p70s6k phosphorylation (representative blot and means ± S.E.M., n=4–6 rats), in soleus muscle strips incubated for 3 min. Values are relative to the respective paired control obtained in the muscle sample incubated in the absence of peptide. LSD test: *P=0·032, †P=0·001.

increment induced by 10^{-10} M GLP-1 being higher than that exerted by 10^{-9} M insulin (Fig. 1).

p70s6k

Figure 2 shows the effect of GLP-1 and that of insulin on p70s6k activity in rat soleus muscle, after a 3 min incubation. GLP-1, at 10^{-10} M, induced a significant increase in the phosphorylation of the enzyme, similar in magnitude to that exerted by 10^{-9} M insulin – with respect to the control value obtained in one or two muscle strips from each experimental rat, incubated in the absence of peptide.

p42/44 MAPKs

Figure 3 shows the action of GLP-1 and that of insulin on p44 and p42 MAPKs activity, in rat soleus muscle after a 3-min incubation. At 10^{-10} M, GLP-1 significantly induced the phosphorylation of both p44 and p42 fractions – with respect to the control value obtained in one or two muscle strips from each experimental rat, incubated in the absence of peptide. The magnitude of this effect of GLP-1 on both MAPKs was statistically indistinguishable from those exerted by 10^{-9} M insulin.

Glycogen synthase a *activation*

Figure 4 shows the effect of wortmannin, rapamycin and PD98059 – considered inhibitors of PI3K, p70s6k and MAPK cellular enzymes respectively – upon the increasing action of 10^{-10} M GLP-1 and 10^{-9} M insulin on glycogen synthase *a* activity in rat muscle strips. As expected, and in the three groups of experiments shown, GLP-1, as well as insulin, induced a significant stimulation

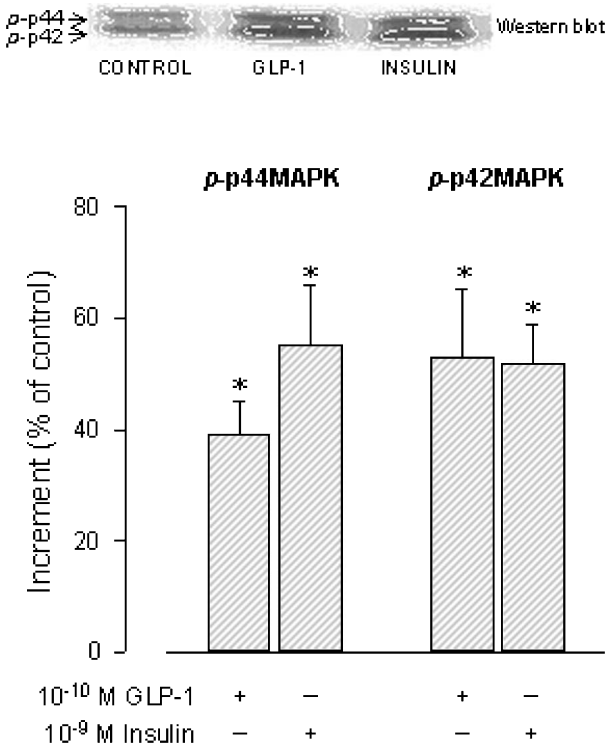


Figure 3 Effect of 10^{-10} M GLP-1 and 10^{-9} M insulin upon phosphorylation of p44 and p42 MAPKs (representative blot and means ± S.E.M. $n=4$–5 rats), in soleus muscle strips incubated for 3 min. Values are relative to the respective paired control obtained in the muscle sample incubated in the absence of peptide. LSD test: $^{*}P<0{\cdot}0001$.

of the enzyme activity, relative to the respective control value obtained in muscle samples incubated in the absence of peptides or inhibitors. The presence of wortmannin dramatically reduced the increase in the glycogen enzyme stimulation exerted by GLP-1, to values even below that of the control (3·23 ± 0·02 mU/g protein, $n=9$ rats), and indistinguishable from that in muscle samples incubated in the sole presence of the inhibitor. Although wortmannin also significantly diminished the insulin-induced stimulation of the enzyme activity, a remaining effect slightly above that of the control was still measured. When p70s6k activity was inhibited by treating the muscle with rapamycin, a drastic reduction of the insulin-induced increase in the activity of glycogen synthase *a* occurred (control: 2·07 ± 0·02 mU/g protein, $n=12$ rats), while that exerted by GLP-1 was not modified. The presence of PD98059 prevented the GLP-1-induced stimulation of the glycogen enzyme activity (control: 2·73 ± 0·03 mU/g protein, $n=10$ rats), but attenuated that of insulin. While the sole presence of rapamycin or PD98059 did not apparently modify the control value, a modest inhibitory effect, although not statistically significant, was measured with wortmannin.

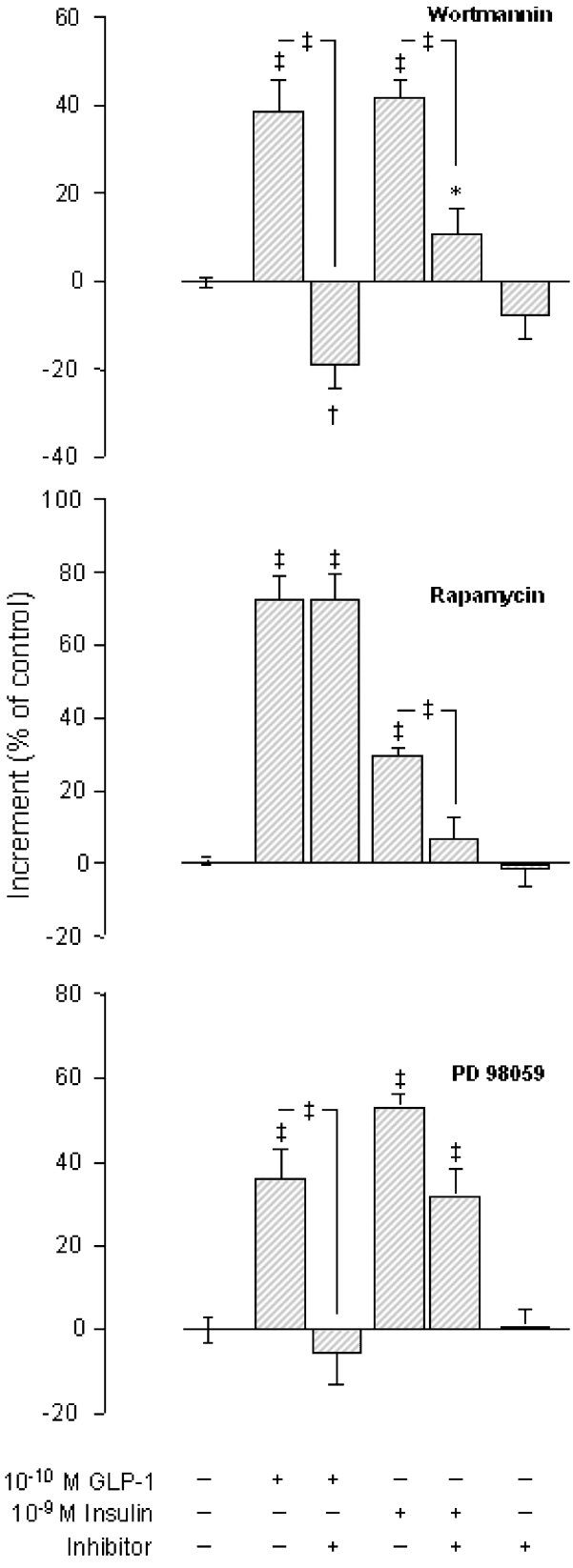
Inhibition of PP-2A activity by okadaic acid treatment (Fig. 5), or that of PP-1 by TNFα, did not modify the GLP-1-induced increment in muscle glycogen synthase *a* activity. Both though reduced that exerted by insulin to similar values to those induced by the respective inhibitor alone, which were apparently lower, particularly in the case of okadaic acid, than that of the corresponding control (control PP-2A: 2·50 ± 0·03 mU/g protein, $n=12$ rats; control PP-1: 2·73 ± 0·03 mU/g protein, $n=8$ rats). When Ro 31–8220 (Fig. 6), a PKC inhibitor, was present, a drastic reduction in the insulin-induced increment of glycogen synthase *a* activity was detected, to a value significantly below the control (1·70 ± 0·03 mU/g protein, $n=14$ rats), and similar to that obtained in the sole presence of the inhibitor. The GLP-1-induced increment in the enzyme activity, which in this group of rats was apparently higher than that exerted by insulin, was partially diminished in muscles treated with Ro 31–8220.

Discussion

We document in this study that GLP-1 activated PI3K/PKB, p70s6k and p42 and p44 MAPKs in normal rat soleus muscle, as it does in freshly isolated rat hepatocytes (Redondo *et al.* 2003), and like the action of insulin in rat hepatocytes (Peak *et al.* 1998) and human myoblasts (Hurel *et al.* 1996).

The stimulatory action of GLP-1 upon muscle glycogen synthase *a* activity was greatly reduced by wortmannin, but not by rapamycin, as occurs in rat hepatocytes (Redondo *et al.* 2003), indicating that activation of PI3K, but not that of p70s6k, is crucial in the action of GLP-1 in these two tissues. The presence of wortmannin also significantly reduced the stimulatory action of insulin, but unlike GLP-1, and as occurs in rat hepatocytes (Redondo *et al.* 2003), rapamycin prevented the stimulatory effect of insulin upon the muscle glycogen enzyme. An inhibitory action of wortmannin in the insulin-induced increase in glycogen synthase *a* activity has already been reported in human myoblasts, in which it was also shown that stimulation of the enzyme by insulin was attenuated by rapamycin (Hurel *et al.* 1996), suggesting that p70s6k could be participating, perhaps not in the initial stimulation of the glycogen enzyme by insulin, but possibly in its maintenance. While some studies in rat hepatocytes have concluded that there is no participation of a rapamycin-sensitive pathway in the control of glycogen synthesis by insulin (Peak *et al.* 1998), others have implicated p70s6k in the rat diaphragm (Azpiazu *et al.* 1996) and also in 3T3-L1 adipocytes (Shepherd *et al.* 1995) but not in rat adipocytes (Moule *et al.* 1995), indicating tissue differences in the insulin-signalling pathways.

Unlike previous observations in rat hepatocytes, in which inhibition of MEK/MAPKs did not affect the GLP-1-induced stimulation of glycogen synthase *a* activity



(Redondo *et al.* 2003), PD98059 in rat muscle completely prevented the increment exerted by the peptide, indicating a role of these kinases in the effect of GLP-1 in the muscle, and also suggesting possible tissue differences in GLP-1-signalling pathways between major physiological targets. Much controversy has been maintained as a consequence of previous data, using various experimental conditions, about the participation of ERKs in the control of glycogen synthase by insulin in extrapancreatic tissues (Dent *et al.* 1990, Cross *et al.* 1995, Lazar *et al.* 1995, Hurel *et al.* 1996, Moxham *et al.* 1996, Peak *et al.* 1998), leading to the general assumption that these kinases are not required, or at least do not play a significant role in the initial activation of the enzyme. Nevertheless, in rat muscle, PD98059 attenuated the increasing action of insulin upon glycogen synthase *a* activity, accompanied by no apparent modification of the control value. This effect, which was similar to that of this MAPK inhibitor on the insulin-induced activation of the glycogen enzyme in isolated rat hepatocytes (Redondo *et al.* 2003), suggests that p44/42 MAPKs are, perhaps, not essential, although they could participate in this action of insulin in those extrapancreatic tissues.

Inhibition of PP-2A by okadaic acid did not affect the GLP-1-induced increment in muscle glycogen synthase *a* (present work), as previously observed in rat hepatocytes (Redondo *et al.* 2003), indicating that this phosphatase does not seem to be required in this action of the peptide. Yet, and unlike its effect upon that of GLP-1, the inhibitor counteracted the action of insulin on the enzyme activity, reducing the value to below even that of the control, and indistinguishable from that obtained in the sole presence of the inhibitor; in liver cells, inhibition of PP-2A modifies neither the control nor the insulin action (Redondo *et al.* 2003). The same was detected whith TNFα – an inhibitor of PP-1 among other actions – suggesting that in the GLP-1-induced increase of rat muscle glycogen *a* synthase activity, activation of PP-1 is not necessary either. As far as we are aware, the regulating mechanism of PP-1 and PP-2A enzymes by insulin and other counter-regulatory hormones is not known, and little has been reported about the role of these two inter-related phosphatases in the control of dephosphorylation of insulin signalling molecules. However, it has been shown that insulin stimulates PP-1 activity in both cultured muscle and fat cells and in mice skeletal muscle (Lazar *et al.* 1995, Moxham *et al.* 1996), and that it has a regulatory role in the glycogen

Figure 4 Glycogen synthase *a* activity in rat soleus muscle strips incubated for 10 min in the absence and presence of 10^{-10} M GLP-1 or 10^{-9} M insulin, and without and with 10^{-6} M wortmannin, 10^{-7} M rapamycin or $2{\cdot}5 \times 10^{-5}$ M PD98059. Values (means ± S.E.M., $n=4$–7 rats) are relative to the respective paired control obtained in the muscle sample incubated in the absence of both peptide and inhibitor (2·78 ± 0·02 mU/g protein, $n=31$ rats). LSD test: *$P=0{\cdot}038$, †$P=0{\cdot}002$, ‡$P<0{\cdot}0001$.

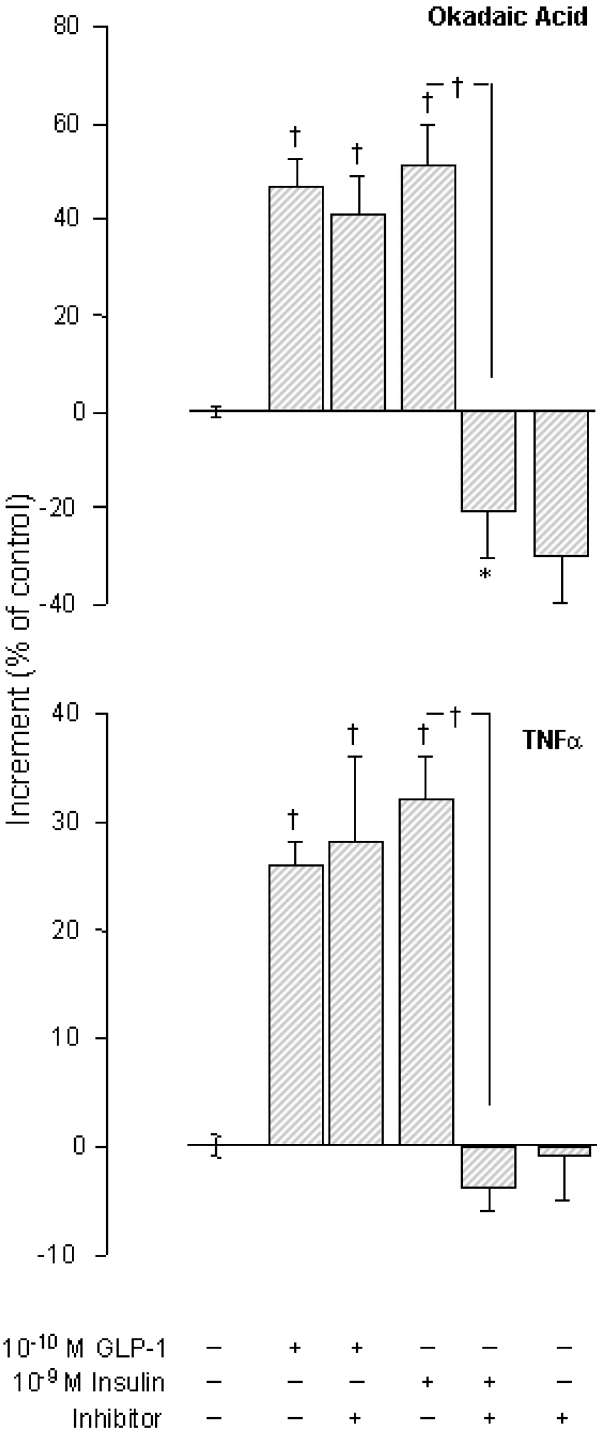


Figure 5 Glycogen synthase *a* activity in rat soleus muscle strips incubated for 10 min in the absence and presence of 10^{-10} M GLP-1 or 10^{-9} M insulin, and without and with 10^{-7} M okadaic acid or 5×10^{-9} M TNFα. Values (means ± S.E.M., n=3–6 rats) are relative to the respective paired control obtained in the muscle sample incubated in the absence of both peptide and inhibitor (2·59 ± 0·02 mU/g protein, n=20 rats). LSD test: *P=0·021, †P<0·0001.

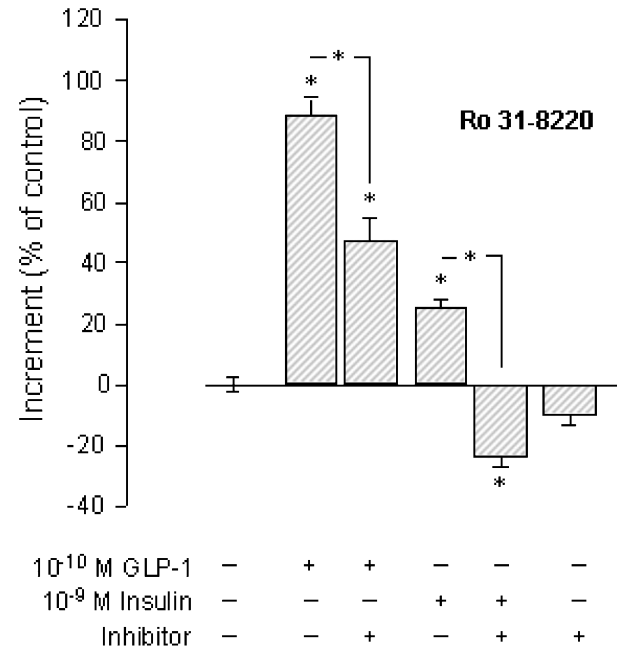


Figure 6 Glycogen synthase *a* activity in rat soleus muscle strips incubated for 10 min in the absence or presence of 10^{-10} M GLP-1 or 10^{-9} M insulin, and without and with 10^{-7} M Ro 31–8220. Values (means ± S.E.M., n=7 rats) are relative to the respective paired control obtained in the muscle sample incubated in the absence of both peptide and inhibitor (1·70 ± 0·03 mU/g protein, n=14 rats). LSD test: *P<0·0001.

synthase activation by insulin in 3T3-L1 adipocytes (Brady *et al.* 1997). Nevertheless, and in relation to the Ser/Thr phosphatase PP-1, our data are in agreement with previous observations in rat muscle and adipose tissue (Ragolia & Begum 1998), from which it was additionally proposed that insulin regulates the activity of PP-1 through a complex cell-specific mechanism that would involve the PKC/PI3K/PKB cascade – with a resultant inhibition of GSK-3 and consequent activation of glycogen synthase, and/or that of ras/MAPKs (Liu & Brautigan 2000); this, despite the fact that results in ovary cells overexpressing insulin receptor made somehow questionable the possibility that PP-1 is required for full activation of the glycogen enzyme by insulin (Liu & Brautigan 2000).

PKC is one of the two Ser/Thr kinases known so far to act downstream from PI3K, PKB being the other; in fact, the atypical PKC isoform ζ has been shown to be activated by the poly-phosphoinositides accumulated in insulin-treated cells, and it has been shown that this kinase is sensitive to PI3K inhibitors such as wortmannin. Also, it has been reported that activation of PKC ζ or PKC λ induces Glut-4 translocation (Pessin & Saltiel 2000), and that several PKC isoforms are chronically activated in human and animal models of insulin resistance (Considine *et al.* 1995, Avignon *et al.* 1996, Ishizuka *et al.* 1998). In addition, PKC has been implicated in the phosphorylation

of GSK-3 and subsequent activation of glycogen synthase *a* in isolated hepatocytes (Pugazhenthi & Khandelwal 1995). In the present study, by using Ro 31–8220, a staurosporine derivative and potent bisindolylmaleimide inhibitor of several PKC isoforms – mainly α, β_1, β_{11}, γ and ε (Wilkinson *et al.* 1993), a moderate, although significant, inhibition of the GLP-1-induced increase in glycogen-synthase *a* activity was detected, as previously observed in rat hepatocytes (Redondo *et al.* 2003). However, the presence of the inhibitor completely blocked the action of insulin in the skeletal muscle, unlike in rat liver cells (Redondo *et al.* 2003), where the stimulatory effect of insulin upon the glycogen enzyme was not apparently modified. Although the present data suggest that PKC might have a role in this action of GLP-1 in the skeletal muscle, no further conclusion can be stated as Ro 31–8220 is not highly selective for any of the different PKC isoenzymes. In fact, the majority of the inhibitors available for the moment, some of them used in this study, could affect more than one protein kinase (Davies *et al.* 2000).

Taking all these factors into consideration, we propose that the stimulatory action of GLP-1 on glycogen synthase *a* in rat skeletal muscle seems to occur through a cellular signalling pathway that would involve PI3K/PKB, MAPKs and perhaps partially PKC, but not the protein phosphatases PP-1 and PP-2A, and as GLP-1 activates the phosphorylation of p70s6k, this kinase could be participating in other effects of GLP-1 in the muscle.

Acknowledgements

This work was supported by grants from the Ministerio de Educación y Cultura (PM 99/0076 and SAF 2002/00938), Spain. We thank Estrella Martín-Crespo for excellent technical assistance and Mark Davis for proof reading the manuscript. A A, N G and V S are Research fellows from the Fundación Conchita Rabago de Jiménez Díaz.

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Received in final form 13 November 2003
Accepted 14 November 2003