

Effects of glucagon-like peptide-1 and exendins on kinase activity, glucose transport and lipid metabolism in adipocytes from normal and type-2 diabetic rats

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Abstract

Several kinases have been implicated in the metabolic response of human and rat myocytes to glucagon-like peptide-1 (GLP-1), exendin-4 (Ex-4) and exendin-9 (Ex-9). We have investigated, in isolated rat adipocytes, the changes caused by GLP-1, Ex-4 and Ex-9 compared with those provoked by insulin or glucagon, upon the activity of phosphatidylinositol-3-kinase (PI3K), protein kinase B (PKB), p42/44 MAP kinases (MAPKs) and p70s6 kinase (p70s6k), and the participation of these kinases and protein kinase C (PKC) in their action upon 2-deoxy-D-glucose uptake, lipolysis and lipogenesis. The study was conducted in normal rats, and extended to a streptozotocin-induced type-2 diabetic model (STZ-rats). The participation of distinct kinases was estimated by using potential kinase inhibitors, including wortmannin, PD98059, rapamycin, H-7 and RO31-8220. In normal rat adipocytes, GLP-1 and both exendins share with insulin an increasing action upon the activity of all kinases studied (except PKB), PI3K, p44 and p42 MAPKs and possibly PKC, all being required for their stimulating effect upon glucose uptake. Ex-4 and Ex-9, like GLP-1 and insulin, have lipogenic action, while only Ex-4 shares with GLP-1 its lipolytic effect which is antagonized by Ex-9. MAP kinases and PKC seem to have an essential role in the GLP-1 and Ex-4 lipolytic action, as does PI3K in that of Ex-4. An increase in PI3K and MAPKs activity for the lipogenic effect of Ex-4, Ex-9 and GLP-1 are required, and in the case of Ex-4 and Ex-9, a stimulation of p70s6k activity is also needed. In cells from STZ-rats the magnitude of the above parameters was, in general, comparable to that in normal animals, with some exceptions: basal PI3K activity and lipogenesis was higher, GLP-1, Ex-4 and Ex-9 failed to modify basal lipogenesis but increased PKB activity, insulin failed to affect the activity of MAPKs and the insulin-induced glucose uptake was impaired. The impaired insulin effects upon some of the variables in the STZ-rat, distinct from those of GLP-1 and exendins, adds knowledge to the mechanism of the beneficial action of GLP-1 and Ex-4 in diabetic states.

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Introduction

Glucagon-like peptide-1 (GLP-1), proposed as a therapeutic tool for type-2 diabetes, has insulin-independent antidiabetic properties (Gutniak *et al.* 1992, D'Alessio *et al.* 1995, Meneilly *et al.* 2001, Dardevet *et al.* 2004) which suggested that GLP-1 should have activity in extrapancreatic tissues participating in overall glucose homeostasis. In fact, it has been demonstrated in several studies that, in liver and muscle, GLP-1 not only has specific receptors (Delgado *et al.* 1995, Villanueva-Peñacarrillo *et al.* 1995), distinct (Valverde *et al.* 1994, Villanueva-Peñacarrillo *et al.* 1994, Yang *et al.* 1998) from those in the pancreas (Thorens 1992), but also that it exerts insulin-like stimulatory effects upon glucose transport and metabolism in normal (Valverde *et al.* 1994, Villanueva-Peñacarrillo *et al.* 1994, Luque *et al.* 2002) and diabetic (Morales *et al.* 1997,

López-Delgado *et al.* 1998) states; in addition, this incretin (Creutzfeldt 2001) modulates the glucose transporter levels in 3T3-L1 adipocytes (Wang *et al.* 1997) and in liver, fat tissue and skeletal muscle of normal and diabetic rats (Villanueva-Peñacarrillo *et al.* 2001*b*).

In fat tissue, where the GLP-1 pancreatic receptor does not seem to be expressed (Bullock *et al.* 1996), the peptide also has specific binding sites (Mérida *et al.* 1993, Valverde *et al.* 1993), and is both lipogenic and lipolytic, apart from showing capability to stimulate parameters related to glucose metabolism (Ruiz-Grande *et al.* 1992, Perea *et al.* 1997, Wang *et al.* 1997, Miki *et al.* 1996, Villanueva-Peñacarrillo *et al.* 2001*a*).

Exendin(1–39)amide (Ex-4), a non mammalian peptide, shares 53% of its amino acid sequence with GLP-1; it is also insulinotropic (Cancelas *et al.* 2001*a*) and exerts GLP-1-like effects in rat liver and skeletal

muscle, such as stimulation of glycogen synthase α , glycogen synthesis and glucose oxidation and utilization (Alcántara *et al.* 1997). Its truncated form, exendin(9–39) amide (Ex-9), has been shown to be an antagonist of the GLP-1 receptor in various cell systems (Nielsen *et al.* 2004), and also of its effects in rat pancreas (Cancelas *et al.* 2001b), liver cells and muscle tissue (Alcántara *et al.* 1997). But in human myocytes, both exendins increase glucose transport (González *et al.* 2005) and metabolism (Luque *et al.* 2002), and have been shown to be agonists of the GLP-1 receptor in adipocyte (Montrose-Rafizadeh *et al.* 1997) and myocyte (Yang *et al.* 1998) cell lines.

GLP-1 increases phosphatidylinositol-3-kinase (PI3K) activity and phosphorylation of protein kinase B (PKB), p44/42 MAP kinases (p44/42 MAPKs) and p70s6 kinase (p70s6k) in human myocytes (González *et al.* 2005) and in rat hepatocytes (Redondo *et al.* 2003) and skeletal muscle (Acitores *et al.* 2004), as insulin does in rat hepatocytes (Redondo *et al.* 2003, Peak *et al.* 1998) and human myocytes (González *et al.* 2005). In human muscle cells, Ex-4 and Ex-9 both share with GLP-1 its stimulatory action on PI3K/PKB and p44/42 MAPK enzymes (González *et al.* 2005) although with an apparent lower potency.

Recent reports have drawn attention to the participation of several kinases in the metabolic response of both human and rat myocytes to GLP-1, Ex-4 and Ex-9 (González *et al.* 2005), and the perturbation of this coupling process in type-2 diabetes mellitus (Acitores *et al.* 2004, González *et al.* 2004). The aim of the present study was to investigate in normal rat adipocytes the effect of GLP-1, Ex-4 and Ex-9, compared with that of insulin or glucagon, on the activity of PI3K, PKB, p70s6k and p42/p44 MAP kinases and the role of these enzymes in the action of GLP-1 and both exendins on 2-deoxy-D-glucose transport and lipid metabolism. As some metabolic responses to insulin are known to be impaired in diabetic states, together with the fact that GLP-1 and Ex-4 are currently under investigation as possible therapeutic tools, we extended the study to adipocytes from adult animals that had been injected with streptozotocin during the neonatal period (STZ-rats) - a current model of type-2 diabetes (Portha *et al.* 1979).

Materials and methods

Reagents

The following reagents were used: human GLP-1(7–36) amide (GLP-1; Bachem AG, Bubendorf, Switzerland); porcine insulin (Novo Biolabs, Bagsvaerd, Denmark); porcine glucagon (Lilly Co., Indianapolis, IN, USA); exendin(1–39)amide (Ex-4) and exendin(9–39)amide (Ex-9) (gifts from Dr John Eng, Veteran's Administration

Medical Center, NY, USA); PD98059 (PD) and RO31–8220 (RO) (Calbiochem, La Jolla, CA, USA); collagenase P (Roche Diagnostics GmbH, Mannheim, Germany); ethylenedinitrilotetraacetic acid (EDTA), bovine serum albumin (BSA), 1-(5-isoquinolinylnsulfonyl)-2-methylpiperazine (H-7), rapamycin (RAP), wortmannin, cytochalasin B, glycerol, phosphatidylinositol and phosphatidylserine (Sigma Chemical Co., St Louis, MO, USA); streptozotocin (STZ) (Sigma-Aldrich Química S.A., Madrid, Spain); aprotinin (Trasylol, Bayer Leverkusen, Germany); 2-deoxy-D-[1,2-³H(N)]glucose (2-DOG; Moravek Biochemicals, Brea, CA, USA); [2-¹⁴C]sodium acetate (Amersham Pharmacia Biotech, UK); dioctyl phthalate (Acros Organics, Fair Lawn, NJ, USA); Ultima Gold scintillation liquid (Packard, Gröninger, The Netherlands); adenosine triphosphate (ATP) and nicotinamide adenine dinucleotide (NAD) (Boehringer Mannheim, S.A., Barcelona, Spain); [γ ³²P]ATP (30 Ci/nmol), horseradish peroxidase-conjugated donkey anti-rabbit immunoglobulin, Rainbow markers, ECL-Western blotting kit, Hyperfilm ECL (Amersham Biosciences, UK); rabbit anti-total and anti-phosphorylated form of p44/42 MAP kinases, p70s6k and PKB (Cell Signaling Technology, New England Biolabs, Beverly, MA, USA); and rabbit anti-PI3-kinase p85 (Upstate Biotechnology, Lake Placid, NY, USA). All other commonly used chemicals were from Sigma or Merck (Merck Pharma Química, S.A., Barcelona, Spain).

Experimental animals

Male Wistar rats, kept on a standard pellet diet (UAR, Panlab, Barcelona, Spain) and tap water which were both available *ad libitum*, were used. Rats were rendered diabetic by a single dose of streptozotocin (STZ, 100 μ g/g body weight (bw)) dissolved in 25 μ l 0.05 M Na citrate, pH 4.5, intraperitoneally administered on the day of birth (Portha *et al.* 1979); at the age of 6–7 weeks, those animals showing a glucose disappearance constant (K) below $2.5 \times 10^{-2} \text{ min}^{-1}$ during an i.v. glucose tolerance test (0.5 mg glucose/g bw, in 30 s) were selected (Vicent *et al.* 1994). A total of 38 STZ-induced type-2 diabetic rats (STZ-rats) were used in this study. Their body weights and K values averaged $257 \pm 9 \text{ g}$ and $1.56 \pm 0.11 \times 10^{-2} \text{ min}^{-1}$ respectively.

Cells

Adipocytes were isolated at 37 °C, by enzymatic digestion with collagenase P, from the rat epididymal fat pads (Rodbell *et al.* 1964). Then, cells were resuspended in Krebs-Ringer Bicarbonate buffer supplemented with HEPES, Trasylol, BSA and without or with D-glucose, pH 7.4, at a density of 10^6 cells/ml.

Kinase activity

Cells (10^6) were first incubated for 15 min in 1 ml KRB containing 30 mM HEPES, 500 KIU/ml Trasylol, 1% BSA, and 3.3 mM D-glucose, pH 7.4, followed by a 3-min incubation in the absence or additional presence of GLP-1, Ex-4, Ex-9 or insulin. For the measurement of PI3K activity and that of phosphorylated PKB, p70s6k and p44/42 MAPKs, the cells were homogenized and maintained at 4 °C in 1.25% Triton containing 250 mM sucrose, 20 mM Tris/HCl, pH 7.6, 2.5 mM MgCl₂, 50 mM 2-mercaptoethanol, 1.2 mM EGTA, 5 mM Na₄P₂O₇, 50 mM NaF, 1 mM Na₃VO₄, 30 U/ml bacitracin, 2 μM leupeptin, 2 μM pepstatin and 2 mM PMSF. After centrifugation at 10 000 *g*, the infranantant, containing cytosol and solubilized membranes, was kept at -70 °C until assay. An aliquot volume was taken from all membrane preparation samples for protein content determination (Bradford 1976).

PI3K activity was estimated as Phosphatidyl Inositol Phosphate (PIP₂) phosphorylation to PIP₃, in p85 immunoprecipitates obtained by treating each adipocyte membrane preparation with anti-PI3-kinase p85 and subsequent coupling to protein A-agarose. The immunoprecipitates were incubated for 20 min at room temperature with 20 μM [γ -³²ATP] (5 μCi/nmol) in 6.25 mM HEPES, 5 mM MgCl₂ and 0.25 mM EGTA, and in the presence of 0.25 mg/ml phosphatidylinositol/phosphatidylserine as substrate; the reaction was interrupted by addition of 400 μl chloroform/methanol/HCl (1:2:1, v/v), 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, v/v), and the new organic phase was separated by centrifugation and then speed-vac dried. The lipidic extract, redissolved in chloroform, was spotted, together with PIP₃ standard, on a silicagel TLC plate, and developed in *n*-propanol/acetic acid/H₂O (66:2:33, v/v). Plates were dried, and radioactive PIP₃ was subsequently visualized by autoradiography and analyzed by densitometric scanning. In all experiments, the densitometric measurement of the band corresponding to cells incubated in the absence of peptide, was used as the control value (Redondo *et al.* 2003).

For the measurements of the degree of phosphorylation of the respective protein kinases by immunoblotting, equal amounts of each solubilized membrane preparation sample were subjected to SDS-PAGE (Laemmli 1970) on an 8% resolving gel, in parallel with molecular weight markers; 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 antibody for each protein

kinase, and a horseradish peroxidase-conjugated donkey anti-rabbit immunoglobulin second antibody; detection was by the enhanced chemiluminescence method, and quantitation was by densitometric scanning of the autoradiography (Redondo *et al.* 2003). The densitometric measurement of the phosphorylated protein kinase was normalized with respect to that of the total (percent of phosphorylated/total kinase), and the value obtained in adipocytes incubated in the absence of peptide was used as the control value.

Glucose transport

Cells (10^5) were incubated for 15 min at 37 °C in 400 μl KRB, 10.9 mM HEPES, 500 KIU/ml Trasylol and 2% BSA, pH 7.4, and either without (basal) or with GLP-1, Ex-4, Ex-9 or insulin alone or combined with 10⁻⁶ M wortmannin (a PI3K inhibitor), 2.5 × 10⁻⁵ M PD (a p44/42 MAPKs inhibitor), 10⁻⁷ M RAP (a p70s6k inhibitor), 10⁻⁴ M H-7 or 10⁻⁷ M RO (PKC inhibitors). This was followed by a 3-min incubation in the additional presence of 0.2 μCi (6.5 pmol) 2-deoxy-D-[1,2-³H(N)]glucose (final concentration 16.3 nM 2-DOG). Adipocytes, after being separated at 10 900 *g* in 100 μl dioctyl phthalate, were added to 3 ml scintillation liquid for β-counting. The total D-glucose content was corrected for the unspecific D-glucose uptake value, obtained in cell samples from each experiment treated in parallel with 0.175 mM cytochalasin B (Perea *et al.* 1997).

Lipolysis

Lipolysis was determined as glycerol release, following Wieland's enzymatic procedure (Wieland 1963), with some modifications (Perea *et al.* 1995). In brief, isolated adipocytes (10^5 cells) were incubated for 60 min at 37 °C, in 300 μl KRB supplemented with 10.9 mM HEPES, 500 KIU/ml Trasylol, 3.3 mM D-glucose and 3% BSA, and in the absence (basal) or presence of GLP-1, Ex-4, Ex-9 or glucagon alone or in combination, and without and with 10⁻⁶ M wortmannin (a PI3K inhibitor), 2.5 × 10⁻⁵ M PD (a p44/42 MAPKs inhibitor), 10⁻⁷ M RAP (a p70s6k inhibitor), 10⁻⁴ M H-7 or 10⁻⁷ M RO (PKC inhibitors); then, 0.45 M HClO₄ was added to the media, and the mixture was maintained for 10 min at 4 °C and centrifuged at 2000 *g*; the supernatant was pH neutralized with 20% KCO₃H, separated at 3000 *g*, and its glycerol content was spectrophotometrically measured as NADH produced in the presence of ATP, NAD and the appropriate enzymes, from the absorption at 340 nm; known amounts of glycerol were used as standards of reference.

Lipogenesis

Lipogenesis was judged from the incorporation of [2-¹⁴C]Na acetate, as precursor, into lipids. Adipocytes

Table 1 Effect of insulin, GLP-1, Ex-4 and Ex-9 on basal kinase activity in adipocytes from normal and STZ-rats. Results are means±S.E.M.

	PI3K	PKB	p70s6k	p42 MAPK	p44 MAPK
Normal rats					
Basal	100 (5)	100 (6)	100 (7)	100 (9)	100 (9)
Insulin (10 ⁻⁹ M)	129±4 (10)*	244±21 (10)*	150±11 (10)*	173±14 (15)*	181±29 (15)*
GLP-1 (10 ⁻¹² M)	105±7 (10)	73±6 (9)	146±13 (8)*	148±9 (14)*	164±13 (14)*
GLP-1 (10 ⁻⁹ M)	143±7 (6)*	75±7 (8)	153±11 (9)*	145±12 (14)*	162±15 (13)*
Ex-4 (10 ⁻⁹ M)	141±4 (6)*	72±12 (5)	171±38 (3)*	142±16 (8)*	162±27 (9)*
Ex-9 (10 ⁻⁹ M)	146±8 (6)*	76±12 (5)	155±27 (3)*	143±11 (9)*	183±20 (8)*
STZ rats					
Basal	100 (4)	100 (6)	100 (3)	100 (6)	100 (6)
Insulin (10 ⁻⁹ M)	137±5 (4)*	284±15 (10)*	199±45 (3)*	77±6 (7)	90±9 (11)
GLP-1 (10 ⁻¹² M)	149±17 (3)*	97±7 (10)	89±1 (3)	88±6 (8)	97±10 (8)
GLP-1 (10 ⁻⁹ M)	135±14 (4)*	173±15 (10)*	163±16 (3)*	138±6 (10)*	154±11 (10)*
Ex-4 (10 ⁻¹² M)	141±19 (3)*	97±10 (5)	—	154±6 (4)*	92±8 (5)
Ex-4 (10 ⁻⁹ M)	142±11 (3)*	167±24 (3)*	109±12 (4)	164±32 (5)*	148±17 (3)*
Ex-9 (10 ⁻¹² M)	91±14 (3)	—	—	120±5 (4)	150±7 (4)*
Ex-9 (10 ⁻⁹ M)	132±3 (3)*	173±31 (5)*	149±8 (3)*	137±14 (3)*	166±15 (3)*

**P*<0.05 vs basal value.

(10⁵ cells) were incubated at 37 °C for 15 min in 400 µl KRB, 10.9 mM HEPES, 500 KIU/ml Trasylol, 3.3 mM D-glucose and 3% BSA, pH 7.4, and without (basal) or with GLP-1, Ex-4, Ex-9 or insulin alone or combined with 10⁻⁶ M wortmannin (PI3K inhibitor), 2.5 × 10⁻⁵ M PD (p44/42 MAPKs inhibitor), 10⁻⁷ M RAP (p70s6k inhibitor), 10⁻⁴ M H-7 or 10⁻⁷ M RO (PKC inhibitors). This was followed by a 60-min incubation at 37 °C in the additional presence of 0.4 mM [2-¹⁴C]Na acetate (0.156 µCi/µmol). Adipocytes, after being separated at 10 900 g in 100 µl diethyl phthalate, were added to 3 ml scintillation liquid for β-counting; the blank value was measured in vials containing all reagents but no cells and subjected in parallel to the same procedure.

Statistical study

All data are presented as mean values (± S.E.M.) together with either the number of individual determinations (*n*) or degrees of freedom (d.f.). The statistical significance of differences were determined using either analysis of variance or the Student's *t*-test.

Results

Effect on kinases activation

In adipocytes from normal rats, the response of the kinases (other than PI3K) to 10⁻¹² M and 10⁻⁹ M GLP-1 was not significantly different; with PI3K, on the other hand, while there was no effect of GLP-1 at the 10⁻¹² M dose, there was a higher and significant (*P*<0.005) increase at 10⁻⁹ M GLP-1 (Table 1). At

10⁻⁹ M, the effect of GLP-1 was not different from that found with either Ex-4 or Ex-9. Pooling together the results obtained with GLP-1 and exendins, the stimulation of enzyme phosphorylation was not different from that found with 10⁻⁹ M insulin in the case of p44 MAPK and p70s6k, it was higher (*P*<0.02) in the case of PI3K, and lower (*P*<0.05) in that of p42 MAPK. Moreover, whilst insulin highly increased PKB activity (*P*<0.001), GLP-1, Ex-4 and Ex-9 inhibited (*P*<0.001) such a phosphorylation to 74 ± 5% (*n*=18) of basal value.

In adipocytes from STZ-rats, the basal activity of the kinases was not significantly different (*P*>0.3) from that found in normal rats, except in the case of PI3K, which averaged 195 ± 9% (*n*=4; *P*<0.005) of that in normal animals. Also, adipocytes from the diabetic rats (Table 1) differed in several other respects. First, whilst insulin (10⁻⁹ M) enhanced PI3K (*P*<0.01), PKB (*P*<0.001) and p70s6k (*P*<0.06) activity as in the normal animals, it failed to affect (*P*>0.7) the phosphorylation of p44 MAPK, and decreased (*P*<0.01) that of p42 MAPK. For PI3K, PKB and p70s6K, the mean readings recorded in the presence of insulin, relative to the paired basal value, were higher, averaging 116.9 ± 6.1% (*n*=17; *P*<0.02) of the mean corresponding value found in normal animals (100.0 ± 3.8%; *n*=30). Secondly, GLP-1, when tested at 10⁻⁹ M, increased (*P*<0.001) PKB phosphorylation, as opposed to the inhibition found in normal rats, while no difference of its effect upon the four other kinases was detected. However, when the concentration of GLP-1 was decreased to 10⁻¹² M, a trend towards an inhibition of PKB, p70s6k, p42 MAPK and p44 MAPK phosphorylation was observed in the STZ-rats, the values averaging 93.7 ± 3.9% (*n*=29; *P*>0.1). This

contrasted with the fact that as little as 10^{-12} M GLP-1 was sufficient to augment ($P<0.05$) the basal PI3K activity. Lastly, in the case of both Ex-4 and Ex-9 (10^{-9} M), an increased phosphorylation ($P<0.01$) of PKB was again observed in the STZ-rats, whilst the effects on the other four kinases were not significantly different from those in normal animals (Table 1).

Uptake of 2-deoxy-D-[1,2- 3 H]glucose

In normal rats, the basal value for D-glucose net uptake averaged 27.2 ± 0.8 fmol/ 10^5 cells ($n=476$). Relative to the mean basal value recorded within the same experiment(s), the readings in the presence of GLP-1, Ex-4 and Ex-9 (all 10^{-9} M) averaged, respectively, $148.5 \pm 3.8\%$ ($n=165$), $144.1 \pm 3.5\%$ ($n=129$) and $147.8 \pm 4.0\%$ ($n=186$); although these values were not significantly different from one another, the overall mean value ($147.0 \pm 2.2\%$; $n=480$) was slightly, but significantly ($P<0.025$) lower than that recorded in the case of insulin ($157.4 \pm 4.1\%$; $n=151$). Likewise, when comparing the readings recorded within the same experiment(s), those obtained in the presence of GLP-1 ($160 \pm 9\%$; $n=46$), Ex-4 ($167 \pm 13\%$; $n=16$) and Ex-9 ($156 \pm 9\%$; $n=57$) yielded an overall mean percentage of $159 \pm 6\%$ ($n=119$), as distinct ($P<0.02$) from $191 \pm 12\%$ ($n=34$) in the presence of insulin. The concentration–response relationships (Fig. 1) for the effect of insulin, GLP-1, Ex-4 and Ex-9 were essentially comparable; thus, the threshold value was close to 10^{-12} to 10^{-11} M, and a maximal response was reached at 10^{-9} to 10^{-8} M.

The basal D-glucose uptake was decreased ($P<0.005$) by PD and H-7 to $89.6 \pm 3.2\%$ ($n=69$) and $87.8 \pm 2.1\%$ ($n=70$) respectively, while in the presence of wortmannin ($93.4 \pm 3.7\%$; $n=93$), RAP ($103.4 \pm 2.3\%$; $n=98$) and RO ($105.4 \pm 1.8\%$; $n=47$), no significant differences compared with the corresponding basal values were found.

As shown in Fig. 2, wortmannin, PD and H-7 abolished, as a rule, the response to insulin, GLP-1, Ex-4 and Ex-9 (10^{-9} M in all cases), the measurements made in the presence of both the agonist and the potential inhibitor being not significantly different from those in the presence of the inhibitor alone. The sole exception was in the case of wortmannin and Ex-4, when the value, although lower ($P<0.001$) than that recorded in the presence of Ex-4 alone, remained somewhat higher ($P<0.001$) than that found in the presence of wortmannin alone. The two other potential inhibitors (RAP and RO) failed to affect significantly the response to insulin, GLP-1, Ex-4 or Ex-9.

In streptozotocin-induced type-2 diabetic rats, three differences became obvious: (1) basal glucose uptake (45.0 ± 2.7 fmol/ 10^5 cells; $n=86$) was higher ($P<0.001$) than that in normal animals; (2) the threshold

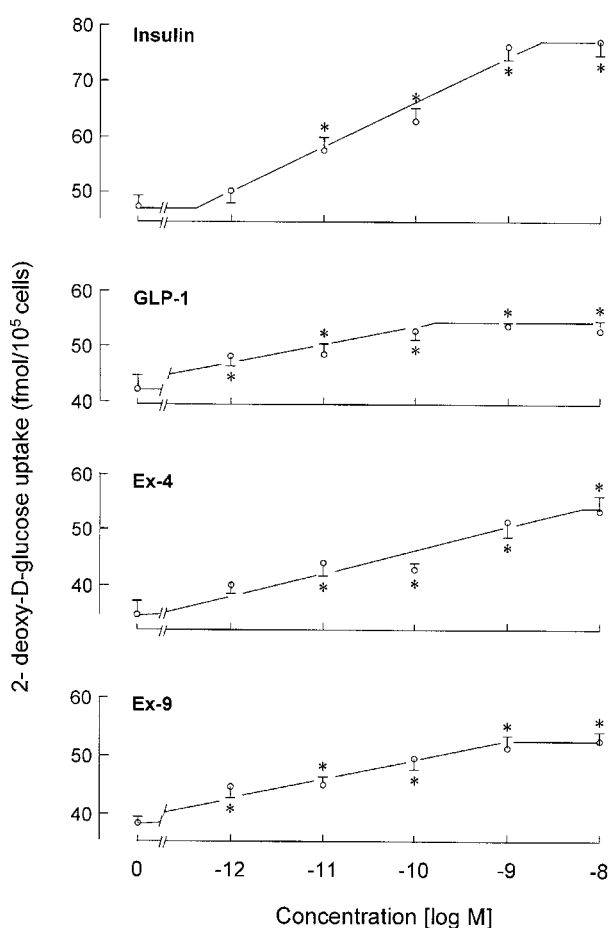


Figure 1 Effects of increasing concentrations of insulin, GLP-1, Ex-4 and Ex-9 on 2-deoxy-D-glucose uptake in normal rat adipocytes. Mean values are expressed relative to the mean basal value found within the same experiment(s), and refer to 11 to 26 separate determinations. * $P<0.05$ compared with the basal value.

stimulation by insulin, but not by GLP-1, Ex-4 and Ex-9, was also higher (10^{-10} M) than in normal rats (10^{-11} M), and (3) at high concentrations (10^{-9} to 10^{-8} M), yielding a close-to-maximal stimulation of glucose uptake, the response to insulin represented only $80.0 \pm 3.1\%$ ($P<0.001$; d.f.=80) of that found in normal rats. Once again, such a decreased responsiveness to insulin was not reproduced with GLP-1, Ex-4 or Ex-9 (10^{-9} to 10^{-8} M), where the results averaged $96.6 \pm 2.3\%$ ($P>0.1$; d.f.=206) of the mean readings recorded in normal animals, a value higher ($P<0.001$) than that found with insulin.

Lipolysis

In normal rats, the basal value for lipolysis averaged 19.2 ± 0.6 nmol glycerol/ 10^5 cells ($n=194$). Both GLP-1

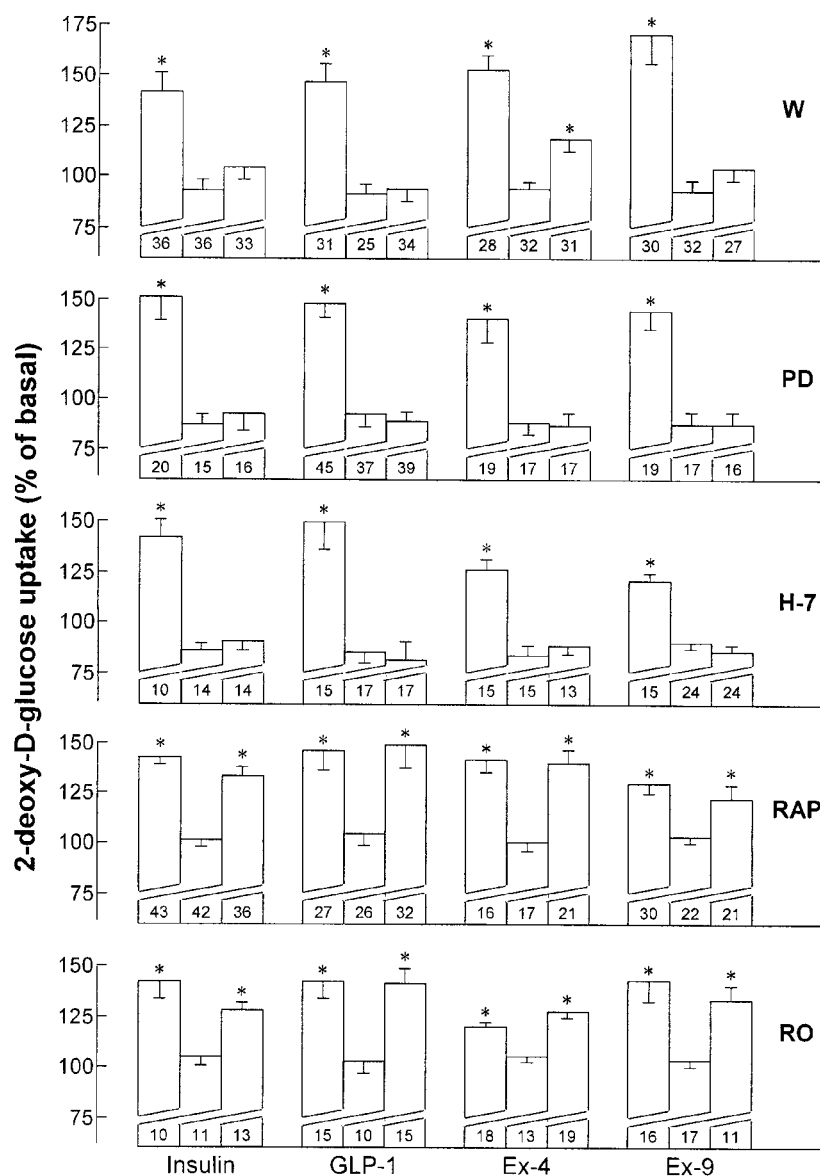


Figure 2 Effects of wortmannin (W), PD, H-7, RAP and RO on 2-deoxy-D-glucose uptake stimulated by insulin, GLP-1, Ex-4 or Ex-9 in normal rat adipocytes. Each group of bars refers to the results found within the same experiment(s) in the presence of the agonist alone (left hand bar), in the presence of the potential inhibitor alone (middle bar) and to the concomitant presence of the agonist and inhibitor (right hand bar). Mean values are expressed relative to the mean basal value found within the same experiment(s), and are derived from the number of separate measurements indicated at the bottom of each bar. * $P < 0.05$ compared with the basal value.

and Ex-4 exerted a stimulatory effect (Fig. 3), with respective threshold concentrations close to 10^{-12} M and 10^{-11} M. The highest mean values recorded at 10^{-9} M GLP-1 ($193 \pm 9\%$; $n=33$) and Ex-4 ($189 \pm 12\%$; $n=22$) were not different from one another. When examined within the same experiment(s), the response to 10^{-9} M GLP-1 did not differ (d.f.=20;

$P > 0.8$) from the response to 10^{-9} M glucagon. At 10^{-11} M, Ex-4 stimulated lipolysis ($P < 0.001$) and increased the response to GLP-1 (10^{-13} , 10^{-12} and 10^{-11} M), the readings recorded in the presence of both agents averaging $113.3 \pm 1.9\%$ ($n=33$; $P < 0.001$) of the corresponding mean values recorded in the presence of GLP-1 alone ($100.0 \pm 1.5\%$; $n=22$); this resulted,

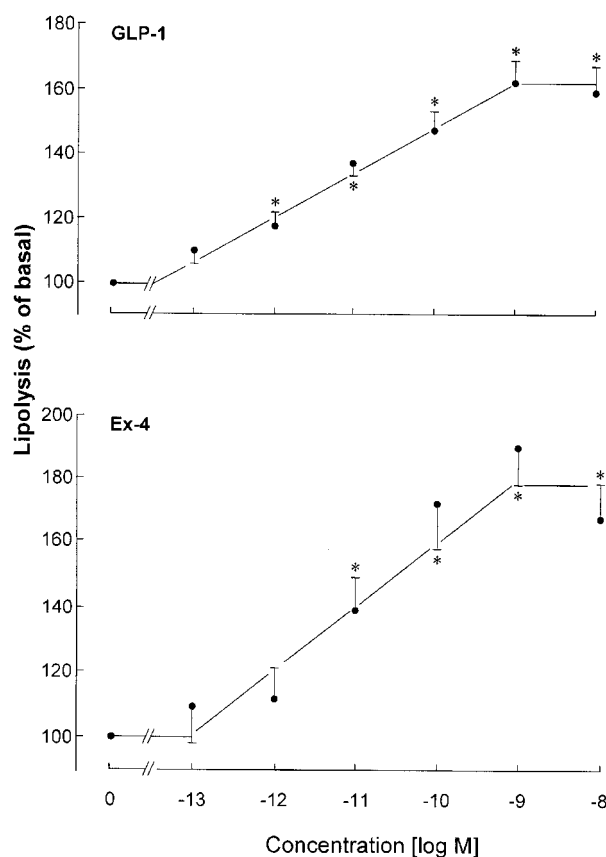


Figure 3 Effects of increasing concentrations of GLP-1 and Ex-4 on lipolysis in normal rat adipocytes. Mean values are expressed as a percentage of the mean basal value found within the same experiment(s), and refer to 10 to 23 separate determinations. * $P < 0.05$ compared with the basal value.

relative to the basal value, in an increment of $17.0 \pm 3.1\%$ (d.f.=61; $P < 0.001$).

No effect of Ex-9 upon lipolysis could be detected at any concentration tested. At 10^{-10} M, however, Ex-9 ($95 \pm 4\%$; $n=37$) suppressed the lipolytic action of 10^{-9} M GLP-1 ($180 \pm 9\%$; $n=37$) and Ex-4 ($159 \pm 7\%$;

$n=38$) to $99 \pm 6\%$ ($n=26$) and $96 \pm 9\%$ ($n=9$) respectively.

Basal lipolysis was not significantly affected by wortmannin, PD, RAP or RO (Table 2) but was decreased ($P < 0.001$) by H-7. All five potential inhibitors reduced ($P < 0.05$) the GLP-1-stimulated lipolysis; however, the values recorded in the presence of both GLP-1 and wortmannin, PD or RAP remained significantly higher ($P < 0.005$) than those obtained in the presence of these agents alone. Such was not the case ($P > 0.1$) with H-7 and RO. The response to Ex-4 was abolished by wortmannin and PD, while RAP, H-7 and RO failed to suppress it, the readings recorded in the presence of these three agents alone averaged $85 \pm 3\%$ ($n=40$; $P < 0.001$) of that found in their absence ($100 \pm 2\%$; $n=39$). In the presence of H-7 and glucagon, lipolysis also remained slightly higher ($P < 0.01$) than that in the presence of the inhibitor alone.

In adipocytes of STZ-diabetic rats, basal lipolysis (25.1 ± 3.0 nmol glycerol/ 10^5 cells; $n=10$) was slightly higher ($P < 0.05$) than that in normal animals, while the response to GLP-1, Ex-4 and glucagon was essentially comparable, averaging $96 \pm 10\%$ ($n=10$) and $163 \pm 11\%$ ($n=10$) in the presence of 10^{-12} and 10^{-9} M GLP-1, $101 \pm 6\%$ ($n=10$) and $144 \pm 10\%$ ($n=10$) in the presence of 10^{-12} and 10^{-9} M Ex-4, and $170 \pm 13\%$ ($n=10$) in the presence of 10^{-9} M glucagon.

Lipogenesis

In adipocytes from normal rats, the basal rate of lipogenesis averaged 2.34 ± 0.15 nmol/ 10^5 cells ($n=214$). It was enhanced (Table 3) by 10^{-9} M GLP-1, Ex-4 and Ex-9 (all $P < 0.001$), these effects were not significantly different from one another ($P > 0.05$), but were significantly lower ($P < 0.001$) than that recorded in the presence of 10^{-9} M insulin. As judged from the concentration/response relationships illustrated in Fig. 4, the threshold concentration was lower with Ex-9 than with Ex-4. In the former case, there was only a very slight trend ($r=0.1724$; d.f.=100; $P < 0.09$) for a positive correlation between the rate of lipogenesis and Ex-9

Table 2 Effect of inhibitors of kinase activity on basal and hormone-stimulated lipolysis in adipocytes from normal rats. Results are means \pm S.E.M.

	Nil	GLP-1 (10^{-9} M)	Ex-4 (10^{-9} M)	Glucagon (10^{-9} M)
Nil	100 \pm 3 (98)	179 \pm 5 (80)*	172 \pm 3 (41)*	181 \pm 6 (22)*
W (10^{-4} M)	99 \pm 4 (39)	126 \pm 6 (29)*	95 \pm 3 (10)	
PD ($2.5 \cdot 10^{-5}$ M)	91 \pm 5 (43)	110 \pm 4 (45)	93 \pm 8 (11)	
RAP (10^{-7} M)	109 \pm 5 (27)	148 \pm 8 (30)*	162 \pm 6 (9)*	
H-7 (10^{-4} M)	88 \pm 2 (18)*	99 \pm 6 (21)	141 \pm 7 (22)*	102 \pm 4 (22)
RO (10^{-7} M)	94 \pm 6 (9)	107 \pm 7 (9)	123 \pm 5 (9)*	

Numbers in parenthesis indicate number of individual determinations.

W, wortmannin.

* $P < 0.006$ vs basal value.

Table 3 Effect of inhibitors of kinase activity on basal and hormone-stimulated lipogenesis in adipocytes from normal rats. Results are means±S.E.M.

	Nil	Insulin (10 ⁻⁹ M)	GLP-1 (10 ⁻⁹ M)	Ex-4 (10 ⁻⁹ M)	Ex-9 (10 ⁻⁹ M)
Nil	100±4 (154)	151±2 (247)*	130±3 (114)*	138±3 (114)*	135±3 (142)*
W (10 ⁻⁶ M)	97±2 (47)	115±3 (20)*	98±3 (22)	102±6 (42)	87±2 (22)*
PD (2.5 10 ⁻⁵ M)	90±2 (47)	126±5 (27)*	89±3 (25)	97±2 (33)	94±2 (35)
RAP (10 ⁻⁷ M)	106±3 (41)	142±4 (17)*	117±7 (16)*	101±3 (40)	98±2 (33)
H-7 (10 ⁻⁴ M)	94±4 (27)	144±11 (34)*	115±7 (33)	104±8 (36)	84±6 (22)
RO (10 ⁻⁷ M)	81±10 (19)	176±18 (21)*	117±12 (21)*	147±14 (20)*	94±9 (20)

Numbers in parenthesis indicate number of individual determinations.

W, wortmannin.

**P*<0.006 vs basal value.

concentration (logarithmic scale). With both Ex-4 and Ex-9, however, no obvious indication of a dual response was observed. Apparently, a concentration of 10⁻⁹ M was sufficient for GLP-1, Ex-4 or Ex-9 to cause a maximal lipogenic response; thus, the effects of 10⁻⁹ M GLP-1 (131±6% of basal value; *n*=18), Ex-4 (134±6%; *n*=16) and Ex-9 (143±8%; *n*=12) were not significantly different from one another or from those recorded in the concomitant presence of GLP-1 and either Ex-4 (137±7%; *n*=15) or Ex-9 (139±11%; *n*=12). Nevertheless, the overall mean value of these five sets of measurements (136±3%; *n*=73) remained significantly lower (*P*<0.02) than that recorded with 10⁻⁹ M insulin (156±9%; *n*=20).

The results recorded in the presence of wortmannin, PD, RAP, H-7 or RO alone were not different (Table 3) from the basal value measured within the same experiment(s). The lipogenic response to 10⁻⁹ M insulin, GLP-1, Ex-4 and Ex-9 was decreased by wortmannin to 85.0±2.3% (*n*=106; *P*<0.001) of the mean corresponding basal value. The rate of lipogenesis in the presence of wortmannin together with GLP-1, Ex-4 or Ex-9 was not different from that in the presence of wortmannin alone, averaging 100.6±3.2% (*n*=86; *P*>0.8) of the latter value; in the concomitant presence of insulin, however, the percentage averaged 117.4±3.1 (*n*=20; *P*<0.001). Likewise, PD abolished the lipogenic response to GLP-1, Ex-4 and Ex-9 with mean readings averaging 101.2±1.5% (*n*=93; *P*>0.4) of those recorded in the presence of PD alone. In the presence of insulin and PD, however, the rate of lipogenesis averaged 89.4±3.6% (*n*=27; *P*<0.05) of that found in the presence of insulin alone, and 150.0±6.0% (*n*=27; *P*<0.001) of that found in the presence of PD alone.

A comparable situation prevailed in the case of RAP, which decreased (*P*<0.001) the response to the agonists under consideration to 83.9±1.6% (*n*=106) of the mean corresponding values found in its absence. In the case of GLP-1, Ex-4 and Ex-9, the residual lipogenesis averaged 97.4±2.0% (*n*=89; *P*>0.15) of that in the

presence of RAP alone; in the case of insulin, however, such a percentage amounted to 134.6±3.8 (*n*=17; *P*<0.001). However, the inhibitory action of RAP on insulin-stimulated lipogenesis failed to achieve statistical significance. Likewise, H-7 failed to affect the response to insulin, at variance with the response to GLP-1, Ex-4 or Ex-9.

Lastly, RO did not significantly decrease the lipogenic response to insulin or even that to GLP-1, whilst that in the presence of both RO and Ex-4, but not RO and Ex-9, remained significantly higher (*P*<0.001) than that recorded in the presence of RO alone.

For all five inhibitors tested in these experiments, the mean highest lipogenic effect, expressed relative to basal value, was always recorded in cells exposed to insulin (140.4±4.9%; *n*=119). Lower readings (*P*<0.001) were always obtained with these inhibitors in the presence of either GLP-1 (106.9±3.3%; *n*=117) or Ex-4 (106.5±3.1%; *n*=171). Lastly, the lowest values were, as a rule, recorded in the presence of Ex-9 (92.2±1.9%; *n*=132), well below (*P*<0.001) that found with either GLP-1 or Ex-4.

In adipocytes from STZ-diabetic rats, major differences were found with those of normal animals: the basal value for lipogenesis (5.01±0.58 nmol/10⁵ cells; *n*=33) was higher (*P*<0.001), and the readings recorded in the presence of 10⁻⁹ M insulin (172±7%; *n*=27), relative to basal value, appeared somewhat higher (*P*<0.01) than in normal rats (151±2%; *n*=247). Also, in sharp contrast to the situation found in adipocytes from normal rats, no enhancing action upon lipogenesis was detected with either GLP-1, Ex-4 or Ex-9, when tested at either 10⁻¹² M or 10⁻⁹ M, the results yielding an overall mean value of 92.5±2.0% (*n*=190), slightly but significantly (*P*<0.001) lower than unity.

Discussion

The present findings support the view that in rat adipocytes PI3K, PKB, p70s6k and p44/42 MAPKs are

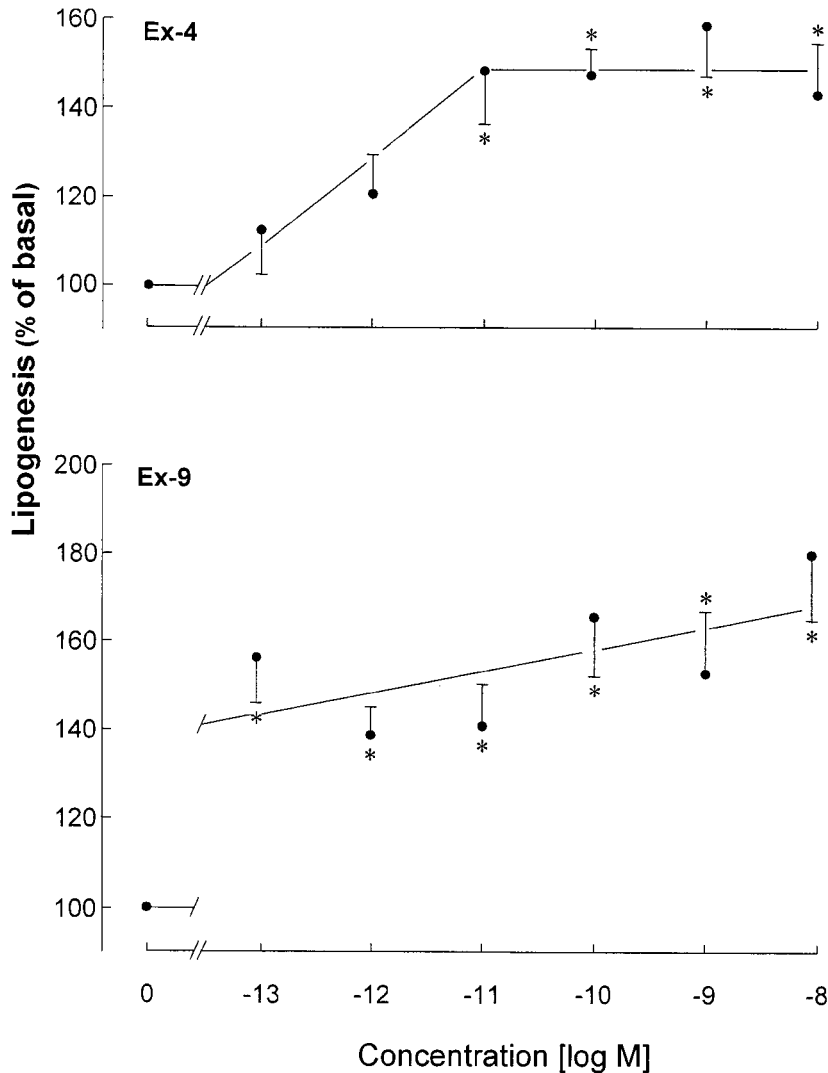


Figure 4 Effects of increasing concentrations of Ex-4 and Ex-9 on lipogenesis in normal rat adipocytes. Mean values are expressed as a percentage of the mean basal value found within the same experiment(s), and refer to 14 to 21 separate determinations. * $P < 0.05$ compared with the basal value.

involved in the metabolic response to GLP-1, Ex-4 and Ex-9; indeed, the activity of these kinases was affected by GLP-1 and both exendins. In normal rats, the responses to GLP-1, Ex-4 and Ex-9 were, generally, similar to one another and were higher than (PI3K), comparable to (p70s6K and p44 MAPK) or less than (p42 MAPK) the response evoked by insulin. In this respect, the most dramatic difference consisted in the enhancement of PKB phosphorylation by insulin, while no increase was detected following GLP-1, Ex-4 or Ex-9; this is in contrast to previous observations in normal rat hepatocytes, in which a clear increment in the enzyme activity evoked by GLP-1, equal in magnitude to that

provoked by insulin, was observed (Redondo *et al.* 2003). The lack of effect of GLP-1, Ex-4 and Ex-9 upon PKB phosphorylation was reversed, however, in the adipocytes of STZ-rats, in which there was also evidence of other anomalies, such as an increased basal PI3K activity, as previously reported in the muscle of type-2 diabetic patients (Meyer *et al.* 2002, González *et al.* 2004), and a suppression of the normal positive effect of insulin on p42 and p44 MAPK phosphorylation. The latter result is in contrast to published observations in the skeletal muscle of rats with STZ-induced diabetes, in which insulin-stimulated ERK2 activity and expression was normal (Markuns *et al.* 1999), and in

agreement with results from myocytes from type-2 diabetic patients (González *et al.* 2004), suggesting not only species but also tissue differences in the hormone-signaling pathway.

The comparable effects of GLP-1 and Ex-4 on enzyme phosphorylation in normal rat adipocytes paralleled the effects on 2-deoxy-D-glucose transport. The same was observed for Ex-9, in contrast with its antagonistic character upon the GLP-1 receptor in the beta-cell and other cell systems (Nielsen *et al.* 2004) and similar to the effect on 3T3 L1 adipocytes (Montrose-Rafizadeh *et al.* 1997) and myocytes (Yang *et al.* 1998). Also it is in accordance with its GLP-1-like action upon glucose transport (González *et al.* 2005) and metabolism (Luque *et al.* 2002) in human myocytes. All this indicates tissue and/or species differences in the effects of Ex-9 and its mechanism of action. Moreover, the potential inhibitors of enzyme activity, wortmannin and PD, abolished, as a rule, the effects of insulin, GLP-1, Ex-4 and Ex-9 on glucose transport, as it occurs in human myocytes (González *et al.* 2005). Such was not the case, however, with RAP, suggesting a more important role for PI3K, MAPKs and possibly PKC than for p70s6K in the control of hexose transport in fat tissue. The postulated involvement of PI3K is further supported by the fact that its increased basal activity in STZ-rats coincided with a higher basal value of 2-deoxy-D-glucose uptake. Likewise, the participation of MAPKs in the regulation of D-glucose transport is consistent with the finding that, in the STZ-rats, the altered effect of insulin on p42 and p44 MAPK phosphorylation occurs together with a preferential impairment of its action on glucose uptake; in fact, insulin, as distinct from GLP-1, Ex-4 and Ex-9, showed both a higher threshold concentration for stimulation of glucose transport and a lower relative magnitude of the metabolic response to higher concentrations of the hormone.

As a general rule, H-7 also abolished the effect of insulin and that of GLP-1, Ex-4 and Ex-9 on D-glucose uptake, as previously observed (except for Ex-9) in human myocytes (González *et al.* 2005). RO, a staurosporine derivative and potent bisindolylmaleimide inhibitor of mainly α , β_I , β_{II} , γ and ϵ PKC isoforms (Wilkinson *et al.* 1993), was ineffective on the action of the four hormones/peptides tested, the effect on that of GLP-1 and insulin coinciding with a previous observation in human myocytes (González *et al.* 2005), and different from that detected on D-glucose metabolism in rat hepatocytes (Redondo *et al.* 2003). Although nothing definite can be concluded as to whether there are one or several PKC isoforms (Davies *et al.* 2000) involved in the respective effect of these hormones/peptides, these data do not exclude the possibility of PKC participating in the cellular signaling mechanism of their action.

A different situation prevailed as far as the regulation of lipolysis in normal rat adipocytes is concerned. Indeed,

GLP-1 and Ex-4, but not Ex-9, stimulated glycerol release, thus mimicking the effect of glucagon rather than that of insulin. Ex-9 opposed the lipolytic action of GLP-1 and Ex-4, in accordance with its antagonistic character on the GLP-1 receptor and its effects in other cell systems (Nielsen *et al.* 2004). Such a lipolytic action was also suppressed, in part at least, by the potential inhibitors of PI3K and MAPKs activity, suggesting that the lipolytic effect of GLP-1 and Ex-4 occurs through activation of either of these two kinases. In adipocytes from STZ-rats, and except for a somewhat higher basal rate of lipolysis (other investigators could not detect any difference in human fat cells (Yu *et al.* 1997)), the lipolytic response to GLP-1, Ex-4 and glucagon was essentially comparable to that in normal rats.

In adipocytes from normal rats, GLP-1, Ex-4 and also Ex-9 again mimicked, albeit to a somewhat lesser relative extent, the positive effect of insulin on lipogenesis, Ex-9, unexpectedly displaying a very low threshold concentration ($\leq 10^{-13}$ M). The sensitivity to the tested inhibitors of kinase activity was lowest in the case of insulin-stimulated lipogenesis and highest in that of Ex-9. Another striking difference between the lipogenic effect of insulin and that of GLP-1 and both exendins consisted in the fact that, in STZ-rats which showed a higher basal value than that in normal animals, no stimulation by GLP-1, Ex-4 and Ex-9 was observed, whilst insulin exerted an increase that was slightly higher than that in normal animals. This more active lipogenic action of insulin in STZ-rats is in accordance with data obtained in type-2 diabetic patients (Lange *et al.* 1988), but is in contrast with a report in a type-2 diabetic mouse model (Hedeskov *et al.* 1992). With respect to the present data, there was a close analogy between the opposite effects of GLP-1, Ex-4 and Ex-9 on both PKB phosphorylation and lipogenesis in normal versus diabetic rats, which may suggest a counter regulatory role for this kinase in the hormonal control of lipid generation. This view is further supported by the finding that the higher relative magnitude of lipogenesis induced by insulin in STZ-rats, as compared with normal animals, coincided with a higher relative increment of PKB, as well as PI3K and p70s6K activity.

In summary, the knowledge that certain enzymes are involved in the metabolic responses of the adipocyte to GLP-1 and exendins in STZ-rats, and the fact that the effect of insulin upon some of them is impaired in this diabetic model, adds further information about the mechanism of the overall beneficial action of GLP-1 and Ex-4, whose effects are being currently studied as an alternative therapeutic tool in human diabetes.

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