Novel Signal Transduction and Peptide Specificity of Glucagon-Like Peptide Receptor in 3T3-L1 Adipocytes

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Glucagon-like peptide-1 (7-36) amide (GLP-1), in addition to its well known effect of enhancing glucose-mediated insulin release, has been shown to have insulinomimetic effects and to enhance insulin-mediated glucose uptake and lipid synthesis in 3T3-L1 adipocytes. To elucidate the mechanisms of GLP-1 action in these cells, we studied the signal transduction and peptide specificity of the GLP-1 response. In 3T3-L1 adipocytes, GLP-1 caused a decrease in intracellular cAMP levels which is the opposite to the response observed in pancreatic beta cells in response to the same peptide. In 3T3-L1 adipocytes, free intracellular calcium was not modified by GLP-1. Peptide specificity was examined to help determine if a different GLP receptor isoform was expressed in 3T3-L1 adipocytes vs. beta cells. Peptides with partial homology to GLP-1 such as GLP-2, GLP-1 (1-36), and glucagon all lowered cAMP levels in 3T3-L1 adipocytes. In addition, an antagonist of pancreatic GLP-1 receptor, exendin-4 (9-39), acted as an agonist to decrease cAMP levels in 3T3-L1 adipocytes as did exendin-4 (1-39), a known agonist for the pancreatic GLP-1 receptor. Binding studies using ^{125}I-GLP-1 also suggest that pancreatic GLP-1 receptor isoform is not responsible for the effect of GLP-1 and related peptides in 3T3-L1 adipocytes. Based on these results, we propose that the major form of the GLP receptor in 3T3-L1 adipocytes is functionally different from the pancreatic GLP-1 receptor. **J. Cell. Physiol. 172:275–283, 1997.**

Glucagon-like peptide (GLP-1) is an incretin hormone secreted from the gastrointestinal endocrine cells in response to food intake. GLP-1 has been proposed to be antidiabetogenic and useful for the treatment of non-insulin dependent diabetes mellitus (Gutniak et al., 1992; Nauck et al., 1993; Nathan et al., 1992). GLP-1 enhances glucose-dependent insulin secretion from pancreatic beta cells (Fehmann et al., 1992; Weir et al., 1989; Montrose-Rafizadeh et al., 1994) and inhibits glucagon secretion from pancreas (Komatsu et al., 1989; Yamato et al., 1990).

In addition to these pancreatic effects, several lines of evidence suggest that GLP-1 has extrapancreatic effects to enhance insulin action. For example, GLP-1 increased insulin sensitivity in both insulin-dependent and non-insulin-dependent diabetic patients (Gutniak et al., 1992). We have shown that GLP-1 enhances insulin-mediated glucose uptake and lipid synthesis in 3T3-L1 adipocytes (Egan et al., 1994). GLP-1 has also been shown to stimulate glycogen synthesis in muscle and liver (Valverde et al., 1994; Villanueva-Penacarrillo et al., 1994), although with respect to muscle this concept has been challenged (Furnsinn et al., 1995).

The endogenous pancreatic GLP-1 receptor has been shown to couple to increases of cAMP production (Gefel et al., 1990; Lu et al., 1993). Transfection of the cloned pancreatic GLP-1 receptor in heterologous expression systems also demonstrated a coupling of this receptor to increases of cAMP production (Widmann et al., 1994) and intracellular Ca^{2+} (Wheeler et al., 1993) levels. However some physiological responses to GLP-1, such as enhancement of lipid synthesis in 3T3-L1 adipocytes (Egan et al., 1994) or stimulation of glycogen synthesis (Valverde et al., 1994; Villanueva-Penacarrillo et al., 1994) in rat muscle and liver, could not be explained by an increase of cAMP or calcium levels, and would suggest the presence of different signal transduction pathways for GLP receptor and/or different GLP receptor isoforms in various tissues. Therefore, we compared

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the signal transduction and peptide specificity of GLP receptor expressed in 3T3-L1 adipocytes with pancreatic GLP-1 receptor overexpression in CHO cells. Our results suggest that GLP-1 and related peptides act in 3T3-L1 adipocytes via functionally different receptors than the pancreatic GLP-1 receptor.

MATERIALS AND METHODS

Plasmid constructs and transfection

Full length GLP-1 receptor cDNA, isolated from rat pancreas (gift from Dr. B. Thorens, University of Lausanne, Switzerland), was subcloned in pSVbeta (Clontec, Palo Alto, CA) downstream of SV40 promoter after replacing beta-galactosidase gene by full length GLP-1 receptor cDNA to obtain pSVGLPR. Full length GLP-1 receptor was also subcloned in a plasmid where GLP-1 receptor and neomycin phosphotransferase (G418 resistance) were each driven by individual mouse RNA polymerase II promoters (pPo12GLPR).

CHO cells that overexpress human insulin receptor (CHO/HIRc) were trypsinized, and resuspended in Ham's-F12 medium containing 10% fetal calf serum. Cells (10^6) were transfected by electroporation (Genepulser, BioRad, Richmond, CA) in a cuvette with a 0.4 cm gap electrode at 300 volts and 960 μF. Cells were cotransfected with 10 μg of HindIII linearized pSVGLPR plasmid and 1 μg of BamH1 linearized pSVHPH (conferring hygromycin resistance) plasmid (American Type Culture Collection, Rockville, MD). As a control, we also transfected the corresponding pSVHPH alone. After electroporation, cells were incubated for 10 min at room temperature, diluted in multiwell plates and incubated overnight at 37°C in a humidified CO_2 atmosphere. Cells were then selected with 700 μg/ml hygromycin for 10 days. The surviving clones were passaged and allowed to propagate in order to obtain enough cells for genomic DNA and RNA preparations. The presence of GLP-1 receptor in genomic DNA and the transcripts were observed in six of eight clones (CHO/pancGLPR) obtained after transfection.

Two micrograms of pPo12GLPR digested with SstII was electroporated into 10^6 3T3-L1 fibroblasts (trypsinized and resuspended in DMEM medium containing 10% calf serum) using identical electroporation conditions as for CHO/HIRc cells. After an overnight incubation, cells were selected with 600 μg/ml (effective concentration) of G418 (Life Technologies, Bethesda, MD) for 7 days. Using this construct all clones that were G418 resistant had GLP-1 receptor expression.

3T3-L1 culture

3T3-L1 fibroblasts were grown (as previously described, Egan et al., 1994) on tissue culture plates in DMEM containing 10% calf serum and maintained in a humidified incubator with 5% CO_2 at 37°C. The cells were induced to differentiate 3 days after reaching confluence by incubation in the presence of DMEM containing 5 μg/ml insulin, 390 ng/ml dexamethasone, 115 μg/ml 3-isobutyl-1-methylxanthine (IBMX), and 10% fetal calf serum for 2 days. The medium was replaced with DMEM containing insulin and 10% fetal bovine serum for an additional 2 days. Thereafter the cells were maintained in DMEM containing 10% fetal bovine serum.

cAMP measurements

Cells grown in 12 well plates were washed three times and incubated with 1 ml of Krebs-Ringer phosphate (KRP) buffer and 0.1% bovine serum albumin (BSA) for 4 hr at 37°C. Cells were then exposed to KRP buffer containing 0.1% BSA and various peptides for 30 min at 37°C (CHO/pancGLPR cells). 3T3-L1 cells were exposed to KRP buffer containing 0.1% BSA, 1 mM IBMX and various different peptides for 90 min at 37°C. Cells were then washed three times with ice-cold Dulbecco's PBS (DPBS), and lysed for 5 min with 1 ml ice-cold 0.6 M perchloric acid. The cell lysate (950 μl) was transferred and pH adjusted to 7.0 using 5 M K_2CO_3. After centrifugation for 5 min at 2,000*g,* the supernatant was aspirated and vacuum dried, and then reconstituted in 500 μl of 500 mM Tris (pH 7.5) and 4 mM EDTA buffer. After addition of 50 μl of 0.15 mM Na_2CO_3 and 50 μl of 0.15 mM $ZnSO_4$, followed by incubation for 15 min on ice, the salt precipitate was removed by centrifugation for 5 min at 2,000*g,* and a 50 μl aliquot of supernatant was assayed using a cAMP [3H] assay kit (Amersham Corp., Arlington Heights, IL). Cellular proteins were solubilized in formic acid and protein content was measured using the Bradford procedure (BioRad, Richmond, CA) and gamma-globulin as standard.

Calcium measurements

Cells attached to glass coverslips were loaded with fura-2 by incubation for 1 hr at 37°C with 6 μM (CHO/pancGLPR cells) or 2 μM (3T3-L1 adipocytes) fura-2-acetoxymethyl ester (fura-2 AM, Molecular Probes, Eugene, OR). For CHO/pancGLPR, cells were incubated in KRP solution without fura-2 for an additional hour at 37°C to increase cleavage of the ester bonds. As previously described (Sterni et al., 1995) the coverslips were mounted in a microscope chamber and perfused at 35°C–37°C with KRP solution containing various reagents. The chamber and microscope objective were heated by jacketed water circulation. Cells were studied on a Zeiss Axiovert microscope with a ×50 Leitz objective. Excitation light was provided by a 75-W xenon lamp attenuated to 20% full power by neutral density filters (Omega). Under these conditions, photobleaching of fura-2 was not detectable. Fura-2 fluorescent emission was measured at 420–570 nm in response to alternating excitation wavelengths of 350 ± 10 nm and 380 ± 10 nm by a computer controlled filter wheel. Pairs of images were collected every 6 sec. Fluorescence was detected by a Hamamatsu intensifier CCD camera (model C2400-97) operating at constant intensifier and camera gain. Data were collected as four frame averages (128 ms/image), which were 8 bit digitized and analyzed by a Perceptics image processor (Knoxville, TN). In each experiment up to 20 cells in the camera field could be selected for real time analysis. Data from each of the selected cells in the field were collected and stored separately and treated as independent observations.

GLP-1 binding measurements

Binding studies were performed on cells plated on 12 well dishes and grown to confluence. Cells were washed in serum free buffer for 2 hr before the binding. Cells

were washed twice with 0.5 ml binding buffer containing 120 mM NaCl, 1.2 mM $MgSO_4$, 15 mM sodium acetate, 5 mM KCl, and 10 mM Tris-HCl, pH 7.6. The cells were then incubated at 4°C for 16 hr with 0.5 ml binding buffer which had added 2% BSA, 500 U/ml aprotinin, 10 mM glucose, a range of concentrations of GLP-1 (0.03–30 nM) and 30,000 cpm ^{125}I-GLP-1 (2000 Ci/mmol, Peninsula, Belmont, CA). We only used freshly prepared ^{125}I-GLP-1 within 2 weeks of reference date. Peptide competition was assessed in the presence of ^{125}I-GLP-1 (0.01 nM) and competing peptides (at 1 nM and 500 nM). At the end of the incubation the supernatant was discarded and the cells were washed three times with 0.5 ml of ice-cold PBS and incubated at room temperature with 0.5 ml of 0.5 N NaOH and 0.1% SDS for 10 min. The radioactivity was measured in the lysates in a ICN Apec Series gamma counter. Specific binding was determined as total binding minus the radioactivity observed in the presence of an excess of unlabelled GLP (500 nM).

Lipolysis assay

Differentiated 3T3-L1 adipocytes in 12 well dishes were washed in Dulbecco's PBS (DPBS) twice and incubated in KRP buffer for 3 hr at 37°C. The buffer was aspirated and replaced with KRP buffer containing 1 unit/ml of adenosine deaminase, 10 nM insulin and 10 nM isoproterenol in the absence or presence of various peptides for 90 min at 37°C. The incubation was terminated by removal of the assay buffer. Glycerol released into the medium was measured by a colorimetric assay (Sigma, St. Louis, MO) or by a fluorimetric method as described previously (Boobis and Maughan, 1983). Similar results were obtained using both methods. The cells were washed three times with ice cold DPBS and lysed with formic acid and total cellular protein was measured in cell lysate aliquots.

RNA preparation, first strand cDNA synthesis and polymerase chain reaction (PCR)

Total cellular RNA was prepared using guanidinium thiocyanate and centrifuging the lysate through a CsCl cushion (Chirgwin et al., 1979; Glisin et al., 1974). Complementary DNA (cDNA) was synthesized from total RNA using Maloney murine leukemia virus reverse transcriptase (Bethesda Research Laboratories, Gaithersburg, MD) and either an oligo(dT) primer or random hexanucleotide primer (Pharmacia LKB Biotechnology Inc., Piscataway, NJ). PCR amplification was performed (Saiki et al., 1988) from first strand cDNA using recombinant Taq DNA polymerase (Amplitaq, Perkin Elmer Cetus). Oligonucleotide primers for glucagon receptor were 5′ primer 5′-TCGACAAGTACTCCTGCTGG-3′ and 3′ primer 5′-ACTTGTAATCAGCATAGTGC-3′ (Svoboda et al., 1993), for GIP receptor were 5′ primer 5′-TTCCGCTGCTACCTGCTTCTTGGCTG-3′ and 3′ primer 5′-CCTCTTTGTTGATGAAGCAGTAGAGC-3′ (Usdin et al., 1993). The oligonucleotide primers for actin mRNA were 5′ primer 5′-TATGGAGAAGATTTGGCACC-3′ and 3′ primer 5′-TCATCGTACTCCTGCTTGC-3′ (Nudel et al., 1983).

Statistical analysis

Results are expressed as the mean ± SEM and subjected to two-tailed Student's paired *t* test.

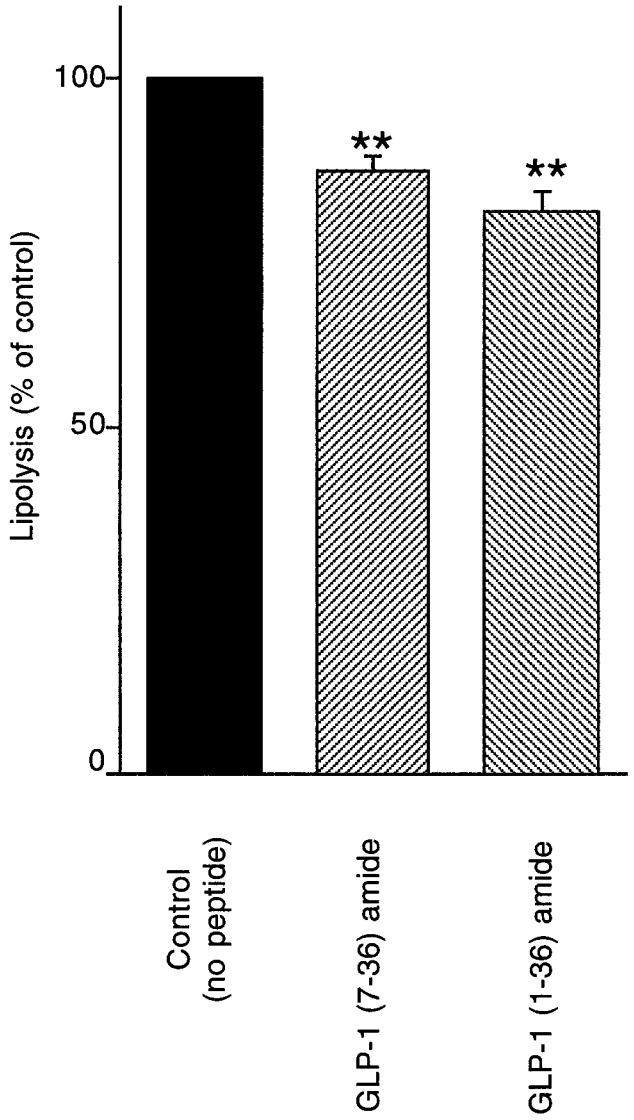

Fig. 1. Effects of GLP-1 on lipolysis in 3T3-L1 adipocytes. 3T3-L1 adipocytes were exposed to 10 nM isoproterenol and 10 nM insulin in the absence or presence of 10 nM of GLP-1 (7-36) amide or GLP-1 (1-36) amide, and glycerol release in the medium was measured. Data are expressed as % of control, i.e. isoproterenol-induced lipolysis in the absence of GLP-1, and are mean ± SEM, of seven experiments performed in triplicate (**$P < 0.01$ vs. control which were 19.9 ± 1.9 μg glycerol/mg protein).

RESULTS

GLP-1 regulates lipolysis in 3T3-L1 adipocytes

To investigate the physiological response to GLP-1 in 3T3-L1 adipocytes, we have shown previously (Egan et al., 1994) that GLP-1 enhances lipid synthesis in 3T3-L1 adipocytes. In this study we measured the effects of 10 nM of GLP-1 (7-36) or GLP-1 (1-36) on isoproterenol (10 nM) induced lipolysis. As shown in Figure 1, GLP-1 (7-36) as well as GLP-1 (1-36) significantly inhibited isoproterenol-induced lipolysis to 86.2 ± 2.6% (mean ± SEM n = 9 $P < 0.01$) and 80.7 ± 3.0% (n = 7, $P < 0.01$), respectively.

Signal transduction of GLP receptor in 3T3-L1 adipocytes versus CHO/pancGLPR cells

To study whether GLP receptor expressed in 3T3-L1 adipocytes was functionally different than pancreatic GLP-1 receptor, we compared the signal transduction linked to GLP receptor in two cell types. Intracellular cAMP content was measured in 3T3-L1 adipocytes or in CHO cells transfected with pancreatic GLP-1 receptor (CHO/pancGLPR). Figure 2A shows that 10 nM GLP-1 (7-36) amide increased the cAMP levels in CHO/pancGLPR cells by 12 ± 4.6-fold (mean ± SEM, n = 3), while GLP-1 (1-36) amide failed to alter cAMP levels. In 3T3-L1 adipocytes, GLP-1 (7-36) amide did not have an effect on basal cAMP levels (data not shown). However as shown in Figure 2B, when 3T3-L1 adipocytes were exposed to 1 mM IBMX (a cAMP phosphodiesterase inhibitor), the cAMP levels increased to 7.2 ± 0.7 pmol cAMP/mg protein. In the presence of IBMX, 10 nM GLP-1 (7-36) amide decreased cAMP to 82.9 ± 4.7% ($P < 0.01$, n = 9) compared to IBMX alone. In similar conditions, GLP-1 (1-36) amide decreased cAMP levels to 83.1 ± 4.3% (mean ± SEM, n = 9, $P < 0.01$). All subsequent experiments to assess cAMP levels in 3T3-L1 adipocytes were performed in the presence of IBMX. Control experiments suggested that the effect of GLP-1 (1-36) amide was not due to its proteolytic conversion to GLP-1 (7-36) amide. This was based on the observation that conditioned medium from 3T3-L1 adipocytes treated with GLP-1 (1-36) amide was ineffective at stimulating cAMP levels when added to CHO/pancGLPR cells (data not shown). Experiments were then performed to assess the dose-dependency of GLP-1 (7-36) amide and GLP-1 (1-36) amide effects on cAMP production. While 1 nM of the peptides were without effect (data not shown), 100 nM elicited a similar effect to 10 nM of peptides, suggesting a saturation of effect. In the presence of IBMX, 100 nM GLP-1 (7-36) amide decreased cAMP levels to 78.6% ± 5.9% (mean ± SEM, n = 4, $P < 0.05$) and GLP-1 (1-36) amide decreased cAMP levels to 81.4% ± 4.7% (mean ± SEM, n = 4, $P < 0.05$) compared to IBMX alone. We did not test higher concentrations of peptides since multiple receptors could be responding to higher concentrations of peptides.

To ascertain the physiological relevance of these modest decreases in intracellular cAMP levels, we also measured changes of cAMP levels in the presence of IBMX and PYY, a known agonist of a G_i-linked receptor. As shown in Figure 2B, 10 nM PYY also significantly decreased cAMP levels to only 80.8 ± 3.8% of control levels (mean ± SEM, n = 7, $P < 0.01$). Thus the observed decrease of intracellular cAMP by GLP-1 is in accord with modest increases (20–40%) of glucose utilization in 3T3-L1 adipocytes (Egan et al., 1994) and may be a consequence of G_i coupling. These results suggest that the GLP receptor in 3T3-L1 adipocytes may be linked to different signal transduction pathways than in CHO/pancGLPR cells.

To investigate whether differences in cell type were responsible for different signal transduction responses to GLP-1 in 3T3-L1 cells vs. CHO/GLPR cells, we transfected pancreatic GLP-1 receptor into 3T3-L1 cells. For unknown reasons, G418 resistant 3T3-L1 clones that overexpressed pancreatic GLP-1 receptor did not differentiate to adipocytes. We therefore compared GLP-1 response in undifferentiated parental 3T3-L1 cells and GLP-1 receptor transfected 3T3-L1 cells. While in the presence of IBMX, 10 nM GLP-1 (7-36) amide had no effect on cAMP levels 96% ± 16% of IBMX control, mean ± SEM, n = 13), in parental undifferentiated 3T3-L1 cells it increased cAMP levels in GLP-1 receptor transfected 3T3-L1 cells by 39 ± 6-fold (mean ± SEM, n = 3) over IBMX control.

The intracellular Ca^{2+} in CHO/pancGLPR cells and in 3T3-L1 adipocytes was also measured by fluorescent imaging of intracellular Fura-2 as a calcium indicator. As shown in Figure 3A, exposure of CHO/pancGLPR cells to 10^{-7} M GLP-1 (7-36) amide induced a transient rise of intracellular Ca^{2+} as monitored by the 350/380 nm fluorescence ratio. In three independent experiments, the ratio of 350/380 nm significantly increased from 0.80 ± 0.02 to a peak value of 1.04 ± 0.04 (mean ± SEM, n = 42 cells, $P < 0.0001$) and then returned to a basal value of 0.82 ± 0.02 over 2 min ($P < 0.0001$ vs. peak value). If ratios are calibrated using pure Fura-2 standards on the microscope stage, the basal free intracellular Ca^{2+} was 83 ± 11 nM and in the presence of GLP-1, increased transiently to a peak value of 239 ± 31 nM and returned to basal value of 90 ± 11 nM. However in 3T3-L1 adipocytes, GLP-1 (7-36) amide (10^{-7} M) had no effect on free intracellular Ca^{2+} concentrations measured in 60 cells in three separate experiments (Fig. 3B). Ionomycin was used as a positive control to confirm the calcium-responsiveness of Fura-2 in each cell. Our data suggest that in contrary to results in CHO/pancGLPR cells, GLP-1 did not affect intracellular free calcium concentrations in 3T3-L1 adipocytes.

Effects of different analogs of GLP-1 in 3T3-L1 cells

A number of peptides related to GLP-1 were used in 3T3-L1 adipocytes to test for their ability to modulate the production of cAMP (Fig. 4). While GLP-2 (10 nM) or glucagon (10 nM) significantly ($P < 0.01$) decreased cAMP production in the presence of IBMX (78.3% ± 5.0% (n = 9) and 76.6% ± 4.4% (n = 7) of IBMX control, respectively), GIP (10 nM) was without effect in 3T3-L1 adipocytes (Fig. 4). On the other hand, 10 nM exendin-4 (1-39), an alternative agonist of pancreatic GLP-1 receptor, did not reduce the levels of cAMP to levels different than IBMX alone (90.4% ± 7.3, n = 7), whereas 100 nM exendin-4 (1-39) produced a statistically significant decrease of cAMP production (82.1% ± 4.6%, n = 4, $P < 0.05$) (Fig. 4). In addition as shown in Figure 4, we tested the effect of 100 nM exendin-4 (9-39), a well defined antagonist of pancreatic GLP-1 receptor and found that, as for GLP-1, it also lowered cAMP levels in 3T3-L1 adipocytes (79.4% ± 5.5, n = 3, $P < 0.05$). Exendin-4 (9-39) did not have an additive effect to GLP-1 on lowering cAMP levels (data not shown). Thus, in contrast to the pancreatic GLP-1 receptor, exendin-4 (9-39) acts as an agonist in 3T3-L1 adipocytes while exendin-4 (1-39) is not as potent an agonist as GLP-1 (7-36) amide. When the effect of these various peptides was assessed on inhibition of isoproterenol-induced lipolysis, we could not reproducibly observe a significant effect on lipolysis (data not shown) suggesting that other mechanisms beside lowering of cAMP are involved in controlling lipolysis.

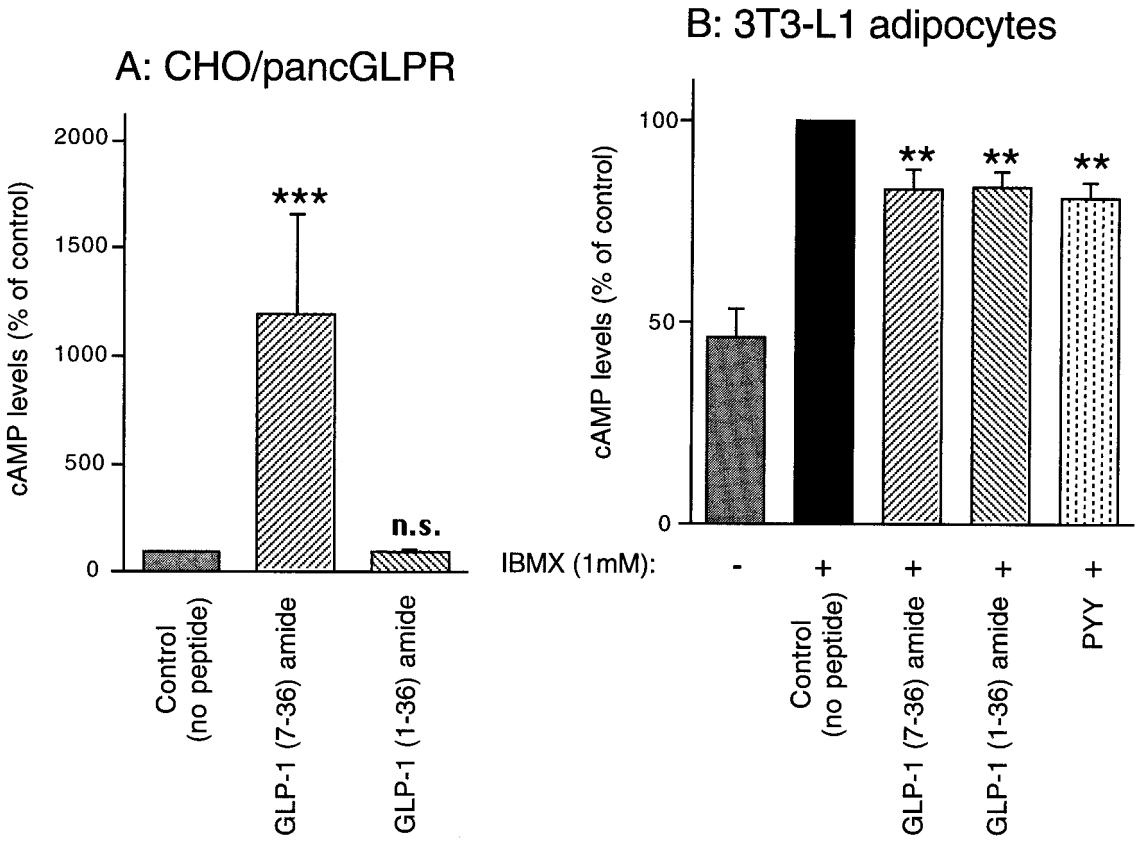

Fig. 2. Effects of GLP-1 on cAMP levels in CHO/pancGLPR (A) and 3T3-L1 adipocytes (B). **A:** Intracellular cAMP levels were measured in CHO/pancGLPR cells after a 30 min incubation in the absence or presence of 10 nM GLP-1 (7-36) amide or GLP-1 (1-36) amide. Data are expressed as % of basal cAMP level and are mean ± SEM of three separate experiments (***$P < 0.001$, n.s. = not significant vs. control). **B:** Intracellular cAMP levels were measured in 3T3-L1 adipocytes after 90 min exposure to 10 nM insulin in the presence or absence of 1 mM IBMX with or without 10 nM GLP-1 (7-36) amide, GLP-1 (1-36) amide or PYY. Data are expressed as % of IBMX response and are mean ± SEM of seven to nine independent experiments performed in duplicate (**$P < 0.01$ vs. IBMX control).

Presence of other peptide receptors in 3T3-L1 adipocytes

To investigate the possibility that GLP-1 could be acting via glucagon or GIP receptors in 3T3-L1 adipocytes (which might have a different signal transduction and/or different peptide specificity), we performed RT-PCR experiments to test for the presence of these receptors in 3T3-L1 adipocytes. Using rat liver glucagon receptor or GIP receptor sequences to design PCR primers, we could not detect the expression of these receptors (Fig. 5) in 3T3-L1 adipocytes even after 60 cycles of PCR. Since 3T3-L1 cells are of mouse origin, results could have been explained by poor cross-species hybridization. However, the predicted size products were observed when mouse pancreas cDNA and mouse liver cDNA were amplified with the primers for GIP receptor and glucagon receptor, respectively (Fig. 5). The integrity of the 3T3-L1 adipocyte cDNA preparations was verified by 30 cycle amplification of actin mRNA. Our data suggest the absence of glucagon receptor or GIP receptor expression in 3T3-L1 adipocytes and suggest that the observed effects of glucagon on cAMP levels are more likely to be via other receptors. We also analyzed mouse epididymal fat RNA for presence of GIP receptor, GLP-1 receptor and glucagon receptor by RT-PCR. As shown previously in rat (Egan et al., 1994; Usdin et al., 1993; Svoboda et al., 1994), mouse epididymal fat expressed low levels of GLP-1 receptor, GIP receptor and glucagon receptor (data not shown). The lack of GIP receptor and glucagon receptor in 3T3-L1 adipocytes shows that 3T3-L1 adipocytes does not mimic epididymal fat. However, lack of these receptors is fortuitous for unmasking the ability of glucagon as well as GLP-1 to couple to different signal transduction pathways.

Next, competition studies were performed with ^{125}I-GLP-1 (7-36) amide in the presence of peptide analogs of GLP-1 in 3T3-L1 adipocytes. GLP-1 (7-36) amide binds to GLP receptor with a Kd of 2.7 ± 0.4 nM and 3,009 ± 589 binding sites per cell (mean ± SEM, n = 3). In these assays, specific binding was 20% ± 2% of total binding, and specific binding was 0.5% ± 0.06% of the total added counts (n = 5, the amount of specific binding was significantly greater than zero, $P < 0.001$). This low percentage of specific binding was found to be a function of low number of GLP-1 binding sites on 3T3-L1 adipocytes, as evidenced by the fact that ^{125}I-GLP-1 (7-36) amide binding in CHO/pancGLPR cells

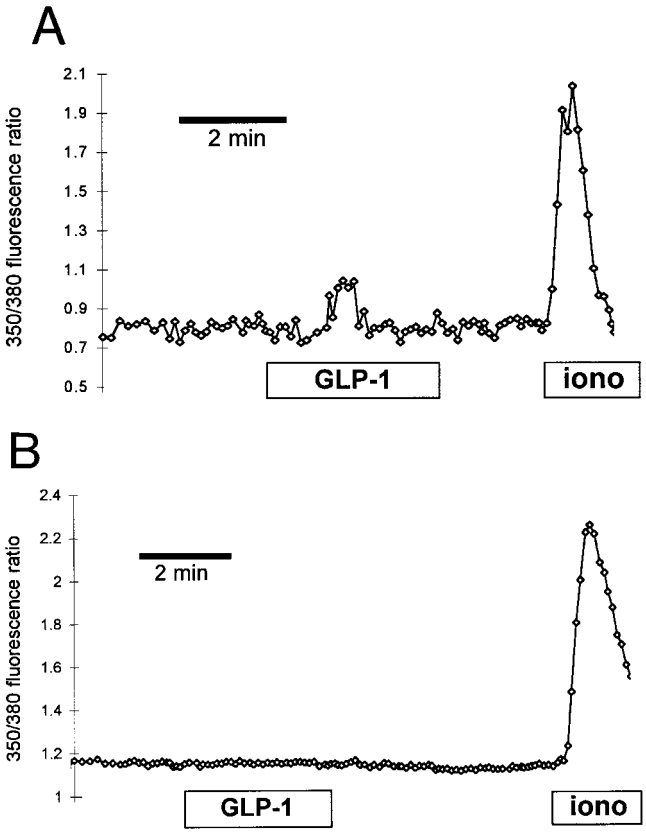

Fig. 3. Effects of GLP-1 on free intracellular calcium in CHO/pancGLPR (A) and in 3T3-L1 adipocytes (B). Free intracellular calcium was measured using a fluorescence imaging microscope in cells attached on coverslips and loaded with Fura-2 dye. The fluorescence ratio of 350 nm/380 nm excitation is shown on the Y axis vs. time. The boxed areas indicate time periods where cells were exposed to either 10^{-7} GLP-1 (7-36) amide (GLP-1) or 3 μM ionomycin (iono) as indicated. **A:** Representative experiment presenting averaged responses of four CHO/pancGLPR cells in a single experiment. Similar results were obtained in three separate experiments. **B:** Representative experiment using 3T3-L1 adipocytes, averaging responses of 20 cells. Similar results were obtained in three separate experiments.

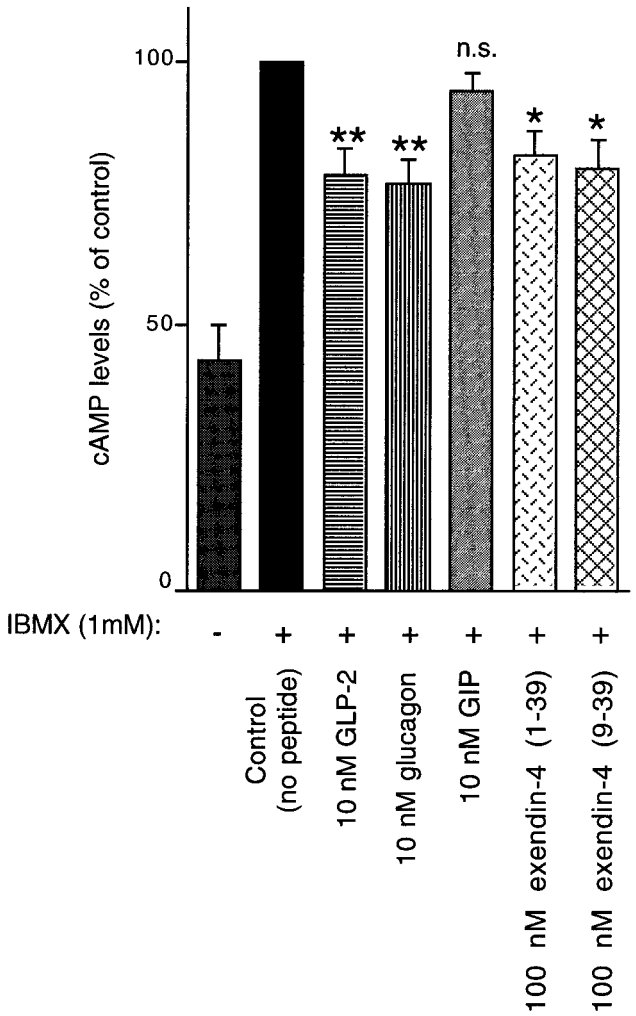

Fig. 4. Effects of GLP-1 analogues on cAMP levels in 3T3-L1 adipocytes. Intracellular cAMP level was measured in 3T3-L1 adipocytes after 90 min incubation with 10 nM insulin and the indicated effectors. Data are expressed as % of IBMX response and are mean ± SEM of four to nine independent experiments performed in duplicate (*$P <$ 0.05 and **$P < 0.01$ vs. IBMX control).

measured 115,000 ± 19,000 binding sites per cell (mean ± SEM, n = 3) with a Kd of 4.6 ± 1.7 nM (n = 3), and yielded specific binding that was only 11% ± 1.7% (n = 3) of the total added counts. We then examined displacement of specific binding of ^{125}I-GLP-1 (7-36) amide in 3T3-L1 adipocytes by 1 nM or 500 nM of various GLP-1 peptide analogs. As shown in Table 1, at 1 nM unlabelled GLP-1 (7-36) amide, glucagon and exendin-4 (9-39) were the only peptides that significantly displaced the ^{125}I-GLP-1 (7-36) amide binding. However, at 500 nM all peptides tested except GLP-1 (1-36) amide competed significantly for ^{125}I-GLP-1 (7-36) amide binding. Interestingly, exendin-4 (1-39) did not compete for GLP-1 binding at 1 nM but competed at 500 nM suggesting lower affinity of this peptide for the 3T3-L1 adipocyte GLP binding site than for pancreatic GLP-1 receptor. In CHO/pancGLPR cells, 1 nM exendin-4 (1-39) competed 85.3% ± 5.3% (mean ± SEM, n = 5) of specific ^{125}I-GLP-1 binding. Since GLP-1 (1-36) amide did not compete with ^{125}I-GLP-1 binding, results suggested that competition by the other peptides at high molar concentrations were not due to nonspecific displacement of binding. The limit of resolution in our binding studies did not allow quantitative affinity measurements of different peptides in 3T3-L1 adipocytes. Our binding data could only detect a receptor qualitatively different in binding characteristics to pancreatic GLP-1 receptor expressed in 3T3-L1 adipocytes. However, the physiological effects of other analogues of GLP-1 observed in these cells implies the likely presence of another receptor for the action of GLP-1 (1-36) amide.

DISCUSSION

An extrapancreatic effect of GLP-1 has been disputed in the literature. Initially Gutniak et al. (1992) showed an increased insulin sensitivity mediated by GLP-1 in patients with NIDDM and patients with IDDM, which suggested an effect of GLP-1 on glucose utilization. In

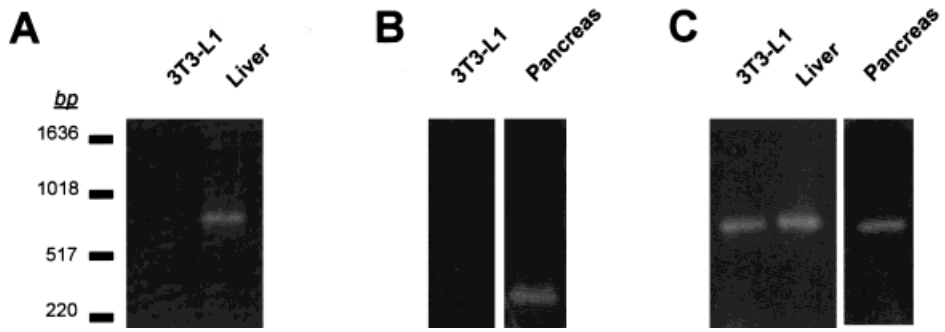

Fig. 5. RT-PCR of glucagon receptor and GIP receptor. Total RNA isolated from mouse liver, mouse pancreas and 3T3-L1 adipocytes were reverse transcribed and amplified by polymerase chain reaction, using specific primers corresponding to **A:** rat glucagon receptor and **B:** GIP receptor Molecular size markers shown on the left are appropriate for both panels. Primers produced a 830 bp amplification product from glucagon receptor of mouse liver and a 300 bp amplification product from the GIP receptor of mouse pancreas. **C:** PCR of actin mRNA from 3T3-L1 adipocytes, mouse liver and mouse pancreas. Primers were designed to produce a 846 bp amplification product from beta actin.

TABLE 1. Inhibition of specific binding of ^{125}I-GLP-1 (7-36) amide by various peptides in 3T3-L1 adipocytes

	Percent displacement of specific binding	
Competing peptide	1 nM	500 nM
GLP-1 (7-36) amide	38 ± 11 (n = 5, *)	100
GLP-1 (1-36) amide	12 ± 10 (n = 4)	29 ± 13 (n = 5)
GLP-2	16 ± 10 (n = 4)	56 ± 17 (n = 5, *)
Glucagon	24 ± 7 (n = 5, *)	46 ± 15 (n = 6, *)
Ex-4 (1-39)	16 ± 13 (n = 3)	87 ± 10 (n = 4, **)
Ex-4 (9-39)	52 ± 7 (n = 3, *)	71 ± 18 (n = 4, *)

Specific binding of ^{125}I-GLP-1 (7-36) amide was competed by various peptides at 1 nM and 500 nM. The data represent percent displacement of specific binding and are mean ± SEM of three to six experiments performed in triplicates. Statistical significance is determined vs. absence of competing peptide (* $P < .05$; ** $P < .01$).

IDDM patients the insulin sensitivity was measured under hyperinsulinemic normoglycemic clamp which minimizes indirect effects of GLP-1 via glucagon on liver glucose output. Similar data were obtained in mice (Ahren, 1995). In addition, D'Alessio et al. (1994, 1995) showed that GLP-1 increases glucose disposal independent of its effect on islet hormone secretion. However several studies challenged these observations and suggested that GLP-1 controls hepatic glucose output and glucose disposal by indirect effects on pancreatic glucoregulatory hormones (Hvidberg et al., 1994; Toft-Nielsen et al., 1996). However, animal and tissue culture models continue to support a direct effect of GLP-1 on glucose disposal (Valverde et al., 1994; Villanueva-Penacarrillo et al., 1994; Tominaga et al., 1996). Presence of GLP-1 receptor has been also observed in various rat and human extrapancreatic tissues (Thorens, 1992; Egan et al., 1994; Delgado et al., 1995; Villanueva-Penacarrillo et al., 1995; Merida et al., 1993).

In this study, we continued our initial observation of direct effect of GLP-1 on glucose utilization in fat cells and further studied the mechanisms underlying these effects. We observed that in 3T3-L1 adipocytes, GLP-1 has a different signal transduction mechanism than pancreatic GLP-1 receptor. While GLP-1 increased cAMP and Ca^{2+} in CHO cells overexpressing pancreatic GLP-1 receptor, GLP-1 did not affect intracellular Ca^{2+} and lowered intracellular cAMP in 3T3-L1 adipocytes. In accordance with this decrease of cAMP, GLP-1 also reversed isoproterenol-induced lipolysis in 3T3-L1 adipocytes. Moreover, when pancreatic GLP-1 receptor was transfected in 3T3-L1 fibroblasts, GLP-1 elicited a pancreatic phenotype of increasing cAMP levels suggesting that sufficient G_S was available for receptor coupling in 3T3-L1 cells. In preliminary experiments, similar results were obtained when pancreatic GLP-1 receptor was expressed in L6 myocytes, where the differentiated GLP-1 transfected myotubes could then couple to increases of cAMP in response to GLP-1 (Yang et al., 1996). Our data suggest that a GLP receptor different than that found in the pancreas is endogenously expressed in 3T3-L1 adipocytes.

We have previously detected the pancreatic GLP-1 receptor sequences in 3T3-L1 adipocytes (Egan et al., 1994), but it remained uncertain whether this cell type expressed full length pancreatic GLP-1 receptor. PCR primers that were able to amplify the pancreatic GLP-1 receptor in all other cell types were ineffective in 3T3-L1 adipocytes, and for unknown reasons we had to use a different set of primers in order to detect the pancreatic GLP-1 receptor sequences in 3T3-L1 adipocytes. Our current work suggests that there is no evidence of functional pancreatic GLP-1 receptor in 3T3-L1 adipocytes.

Results suggested that GLP-1 (1-36) amide and GLP-2 are acting via pathways that are partially or wholly independent of GLP-1 (7-36) amide. In addition to GLP-1 (7-36) amide, GLP-1 (1-36) amide also lowered cAMP levels and inhibited isoproterenol-induced lipolysis in 3T3-L1 adipocytes. This is in accordance with previous studies where GLP-1 (1-36) and GLP-1 (7-36) were equally effective in stimulating glycogen synthesis (Valverde et al., 1994). However, in contrast to previous studies (Delgado et al., 1995), GLP-1 (1-36) amide did not compete for ^{125}I-GLP-1 binding suggesting they were not acting at the same receptor. GLP-2 is a peptide with 50% sequence homology to GLP-1 and glucagon and is processed to be released by intestinal endocrine cells after food intake (Fehmann et al., 1995; Mojsov

et al., 1986). GLP-2 has been recently shown to be involved in hexose transport in rat intestine (Cheeseman et al., 1996) as well as in mice intestinal proliferation (Drucker et al., 1996). In this study, we observed that GLP-2 also lowered cAMP levels and competed with low affinity for GLP-1 (7-36) amide binding.

Evidence suggests that some GLP-1 receptor agonists in 3T3-L1 adipocytes are working via mechanisms other than the known pancreatic beta cells GLP-1 receptor. In 3T3-L1 adipocytes, exendin-4 (9-39) acted as agonist, and exendin-4 (1-39) had much lower potency vs. GLP-1 (7-36) amide: both effects are different vs. the known pancreatic GLP-1 receptor. In addition, the GLP receptor in 3T3-L1 adipocytes binds well glucagon which is different than the pancreatic GLP-1 receptor. Several lines of evidence rule out the possibility that the effects observed could be via the glucagon receptor. First, no glucagon receptor was detected by 60 cycles PCR. Second, glucagon lowers cAMP in these cells implying that the glucagon effect is mediated via a receptor functionally different from the known liver glucagon receptor. Lastly, the displacement of ^{125}I-GLP-1 (7-36) amide binding by glucagon or unlabelled GLP-1 (7-36) amide was comparable and with high affinity which is different than known interactions of GLP-1 (7-36) amide and glucagon peptides at their respective pancreatic receptors (Fehmann et al., 1995). In accord with our data, other experiments have suggested that the extrapancreatic effects of GLP-1 in liver, skeletal muscle and adipose tissues could be via GLP-1 binding to as yet unidentified receptor (Bullock et al., 1996).

In summary, our data suggest either that there is a different receptor-mediated signal tranduction mechanism for GLP-1 and GLP-1 analogs in 3T3-L1 adipocytes and/or that 3T3-L1 adipocytes express a novel non-pancreatic GLP receptor isoform.

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