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Signal Transduction by the Cloned Glucagon-like Peptide-1 Receptor: Comparison with Signaling by the Endogenous Receptors of β Cell Lines

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SUMMARY

Glucagon-like peptide-1 (GLP-1) is a gastrointestinal hormone that potentiates glucose-induced insulin secretion by pancreatic β cells. The mechanisms of interaction between GLP-1 and glucose signaling pathways are not well understood. Here we studied the coupling of the cloned GLP-1 receptor, expressed in fibroblasts or in COS cells, to intracellular second messengers and compared this signaling with that of the endogenous receptor expressed in insulinoma cell lines. Binding of GLP-1 to the cloned receptor stimulated formation of cAMP with the same dose dependence and similar kinetics, compared with the endogenous receptor of insulinoma cells. Compared with forskolin-induced cAMP accumulation, that induced by GLP-1 proceeded with the same initial kinetics but rapidly reached a plateau, suggesting fast desensitization of the receptor. Coupling to the phospholipase C pathway was assessed by measuring inositol phosphate production and variations in the intracellular calcium concentration. No GLP-1-induced production of inositol phosphates could be measured in the different cell types studied. A rise in the intracellular calcium concentration was nevertheless observed in transfected COS cells but was much smaller than that observed in response to norepinephrine in cells also expressing the α_{1B}-adrenergic receptor. Importantly, no such increase in the intracellular calcium concentration could be observed in transfected fibroblasts or insulinoma cells, which, however, responded well to thrombin or carbachol, respectively. Together, our data show that interaction between GLP-1 and glucose signaling pathways in β cells may be mediated uniquely by an increase in the intracellular cAMP concentration, with the consequent activation of protein kinase A and phosphorylation of elements of the glucose-sensing apparatus or of the insulin granule exocytic machinery.

In response to nutrient absorption, in particular carbohydrates, intestinal endocrine cells secrete peptidic hormones that potentiate glucose-induced insulin secretion by pancreatic β cells. These hormones are collectively called gluco-incretins (1–3). One of the most potent gluco-incretin characterized so far is GLP-1 (4). This hormone, also referred to as truncated GLP-1, GLP-1-(7–36) amide, or GLP-1-(7–37), is derived by proteolytic processing of the preproglucagon molecule in intestinal L cells and corresponds to residues 78–107 of this precursor molecule (5, 6). Specific cDNAs for a GLP-1 receptor have recently been isolated and characterized by screening of rat (7) and human (8) pancreatic islet cDNA libraries. This receptor belongs to the secretin/glucagon receptor subfamily of seven-transmembrane segment receptors that are all coupled to heterotrimeric G proteins.

Gluco-incretin hormones are not secretagogues by themselves and only potentiate insulin secretion in the presence of normal or elevated extracellular glucose concentrations (9). This implies that signal transduction by the GLP-1 receptor interferes with the glucose signaling pathway (10, 11). The mechanisms of this interaction are not well understood. However, coupling of the receptor to adenylyl cyclase has been demonstrated (7, 8, 12, 13) and therefore interaction of both signaling pathways is probably mediated, at least in part, by activation of protein kinase A. Possible targets of this kinase may include ATP-dependent potassium channels, voltage-activated calcium channels, and elements of the exocytic machinery itself (14–16). Contributions by other second messengers could also be expected to occur, because some of the receptors related to the GLP-1 receptor, such as those for calcitonin and parathyroid hormone (17, 18), are coupled to both the adenylyl

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ABBREVIATIONS: GLP-1, glucagon-like peptide-1; CHL, Chinese hamster lung; FCS, fetal calf serum; DMEM, Dulbecco's modified Eagle medium; AFB, ammonium formate buffer; IBMX, isobutylmethylxanthine; HBS, HEPES-buffered saline; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

cyclase and phospholipase C pathways. Furthermore, a recent report described the coupling of the cloned GLP-1 receptor to the phospholipase C pathway when the receptor was overexpressed in COS cells (13). Because a rise in intracellular calcium concentration is the eventual signal generated by glucose stimulation of β cells that leads to insulin secretion (19), it is important to assess whether the coupling to intracellular calcium observed in COS cells is physiologically significant in the control of insulin secretion by β cells.

In the present work we studied the signaling pathways activated by GLP-1 binding to its receptor, which was expressed from the cloned cDNA in transfected COS cells and fibroblasts. In addition, we compared this coupling with that of the endogenous receptor present in different insulinoma cell lines. Our data show that coupling of the receptor to adenylyl cyclase occurred with the same efficiency and kinetics in these three different cell types. In contrast, GLP-1 did not induce significant inositol phosphate production in any cell type. Although a small increase in the intracellular calcium concentration was measured in transiently transfected COS cells, no such intracellular calcium mobilization could be observed in stably transfected fibroblasts or in the different insulinoma cell lines studied. Together, our data show that in insulinomas, and therefore probably also in pancreatic β cells, interaction between the GLP-1 and glucose signaling pathways is mediated only by activation of adenylyl cyclase, with the consequent rise in the intracellular cAMP concentration and activation of protein kinase A. Furthermore, because coupling of the receptor to second messengers in transfected fibroblasts is similar to that in insulinoma cells, our data indicate that this cell line can be used for further biochemical studies of the receptor.

Materials and Methods

Cells and cell culture. The INS-1 insulinoma cell line (20) was a gift of Dr. M. Asfari, Hôpital Saint-Antoine, Paris, the RIN 1027-B2 and 1056A cell lines (21) were provided by Dr. Jacques Philippe, University of Geneva, and the βTC3 cells were from S. Efrat, Albert Einstein University, Bronx (22). The cells were cultured in RPMI 1640 medium, 10% FCS, 2 mM glutamine, 1 mM sodium pyruvate, 50 μM β-mercaptoethanol, 50 IU/ml streptomycin/penicillin, 10 mM HEPES, 2 g/liter $NaHCO_3$, pH 7.2. COS cells and CHL fibroblasts (CCL39; American Type Culture Collection) were grown in DMEM with 10% FCS, 2 mM glutamine, and 50 IU/ml streptomycin/penicillin (DMEM-c).

Transformation of COS cells. COS cells (4×10^5) were plated in 100-mm tissue culture dishes. Twenty-four hours later, they were washed once with 5 ml of phosphate-buffered saline and incubated in 4 ml of transformation medium (DMEM, 10% NuSerum, 20 mM HEPES, pH 7.4, 2 mM glutamine, 5 IU/ml penicillin/streptomycin, 100 μM chloroquine, 400 μg/ml DEAE-dextran) containing 4 μg of DNA (23). DNAs used for COS cell transformation were pGLPR-1 (encoding the rat GLP-1 receptor) (7), pcDNA-1 (plasmid vector used to construct pGLPR-1) (Invitrogen), and pBC12BI-α1B (encoding the α_{1B}-adrenergic receptor) (24). Cells were then incubated at 37° in 5% CO_2 for 2–4 hr, washed with 5 ml of DMEM containing 10% dimethylsulfoxide, and incubated for 2–3 days in DMEM-c before analysis.

Stable CCL39 transformants. CHL cells at 10–20% confluency were cotransfected, by the calcium phosphate precipitation procedure, with plasmid containing the GLP-1 receptor (plasmid pGLPR-1) (7) and the pWLneo plasmid (Stratagene) containing the neomycin resistance gene, at a ratio of 10:1. Clones resistant to 0.8 mg/ml neomycin (Geneticin; GIBCO/BRL) were isolated by trypsinization using 5-mm cloning rings and were amplified in neomycin-containing DMEM-c before binding analysis.

Binding analysis. Iodination of GLP-1 and binding of iodinated GLP-1 were carried out as described earlier (7, 8). Determinations of affinity constants (K_d) and number of receptors expressed per cell (B_{max}) were derived from Scatchard plot analysis of experiments performed with the INS-1 cell line, COS cells, and CHL fibroblasts, with the latter two being transformed with the cDNA encoding the rat GLP-1 receptor. Using β-galactosidase as a reporter gene, transformation efficiency in COS cells has been estimated to be about 10% (data not shown). This transformation efficiency was taken into account in calculations of the B_{max} of COS cells expressing wild-type rat GLP-1 receptors.

cAMP assay on intact cells. Cells grown for 2 days in 12-well plates were loaded with 2 μCi of tritiated adenine (no. TRK311; Amersham) in 1 ml of DMEM, with 0.5% FCS, for 5–6 hr at 37°. Cells were then washed twice with HBS (130 mM NaCl, 20 mM HEPES, pH 7.4, 0.9 mM NaH_2PO_4, 0.8 mM $MgSO_4$, 5.4 mM KCl, 1.8 mM $CaCl_2$, 25 mM glucose, 25 μM phenol red) and exposed to GLP-1 or forskolin in HBS, in the presence of 1 mM IBMX, for 8–15 min at 37°. For intracellular cAMP measurements, supernatants were discarded and the cells were lysed with 1 ml of stop solution (5% trichloroacetic acid, 0.1 mM unlabeled ATP, 0.1 mM unlabeled cAMP). For total cAMP measurements, supernatants were not discarded and an equal volume of stop solution was added. Control experiments demonstrated that 85% of cAMP produced by stimulated GLP-1 receptor-expressing fibroblasts remained intracellular (data not shown). Tritiated cAMP was sequentially separated on Dowex cation exchange resin and aluminium oxide columns, as described (25).

Adenylyl cyclase assay on membrane preparations. Cells grown for 2 days in 10-mm-diameter culture dishes were washed twice with phosphate-buffered saline. Cells were scraped with a rubber policeman in 1 ml of TH solution (5 mM Tris·HCl, pH 7.4, 5 mM EDTA). One milliliter of TH solution was used to rinse the culture dishes. The cells were then centrifuged at 50,000 × *g* for 30 min at 4°. The pellet was resuspended in TI solution (50 mM Tris·HCl, pH 7.4, 5 mM EDTA, 10 mM $MgCl_2$) (200 μl of TI solution/pellet obtained from one culture dish). The protein concentration of the crude membrane suspension was then determined with a bicinchoninic acid assay, according to the manufacturer's protocol (no. 23223; Pierce), using bovine serum albumin as standard. Adenylyl cyclase activity was determined by the method of Salomon *et al.* (26). Assay mixtures contained 20 μl of crude membrane suspension (30–50 μg of proteins), 30 mM Tris·HCl, pH 7.4, 6 mM $MgCl_2$, 3 mM EDTA, 120 μM ATP (no. A5394; Sigma), 1 μCi of [α-^{32}P]ATP (no. NEG003; NEN), 100 μM cAMP (no. A4137; Sigma), 53 μM GTP (no. G5631; Sigma), 2.8 mM phosphoenol pyruvate (no. P7252; Sigma), 20 units/ml myokinase (no. M3003; Sigma), 4 units/ml pyruvate kinase (no. 5506; Calbiochem), and 10 μl of 5× concentrated agonists, in a final volume of 50 μl. Reactions were initiated by the addition of crude membranes and the assay mixtures were incubated for 60 min at 37°. Reactions were terminated by the addition of 1 ml of 0.2 mg/ml ATP and 0.1 mg/ml cAMP, and cAMP was isolated sequentially on Dowex cation exchange resin and aluminium oxide columns, as described (25). Determinations were performed in triplicate.

Measurement of phosphoinositide breakdown. Cells grown for 2 days in 12-well plates were loaded with 2 μCi of tritiated inositol (TRK911; Amersham) in 1 ml of DMEM, with 0.5% FCS, for 16–18 hr at 37°. Cells were then washed twice with HBS and stimulated with the indicated agonists for 20 min at 37° in HBS with 20 mM LiCl. The supernatant was discarded and the cells were lysed for 30 min at 4° with 0.75 ml of 10 mM HCOOH. Three milliliters of 3 mM NH_3 were loaded onto Dowex columns (1-ml bed volume of AG1-X8, 200–400 mesh; Bio-Rad), immediately followed by loading of the cell lysates. Columns were washed once with 4 ml of AFB (40 mM NH_4COOH, adjusted to pH 5 with formic acid). Inositol phosphates were eluted with 4 ml of 2 M AFB and the radioactivity was measured in a β-counter after addition of 9 ml of liquid scintillation cocktail (Emulsifier

Scintillatorplus; Packard). Columns were regenerated with 2 ml of 2 M AFB, 10 ml of water, and 2 ml of 1.5 mM NH_3.

Intracellular calcium concentrations. Cells grown in 10-cm culture dishes were harvested by trypsinization, washed once, and resuspended in 3 ml of physiological salt solution (140 mM NaCl, 4.6 mM KCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 10 mM glucose, 10 mM HEPES, pH 7.4). The cells were loaded with 2.5 μM concentrations of the acetoxymethyl ester form of the calcium-sensitive dye fura-2 (Boehringer Mannheim) for 30 min at room temperature in the dark. The dye was added from a 1 mM stock solution in dry dimethylsulfoxide. Cells were then washed twice and resuspended in 2 ml of physiological salt solution. Intracellular calcium concentrations were determined at room temperature with a Perkin-Elmer LS50 spectrofluorimeter, using dedicated software (Intracellular Biochemistry; Perkin-Elmer). Excitation wavelengths for the calcium-bound and free dye were 340 and 380 nm, respectively. Slit openings in both the excitation and emission paths were set at 7.5 nm. Emission signals were collected at 505 nm and automatically ratioed (340 nm/380 nm) every 2 sec. The calcium concentrations were calibrated after each single experiment by determining the maximal (F_{max}) and minimal (F_{min}) fluorescence ratios. F_{max} and F_{min} were measured by first lysing the cells with Triton X-100 (0.1%) and then chelating total calcium with 25 mM EDTA. Absolute calcium concentrations were then calculated by the software using the method described by Grynkiewicz *et al.* (27), according to the following equation: $[Ca^{2+}]_i = K_d \times [(F - F_{min})/(F_{max} - F)] \times (S_{f2}/S_{b2})$, where K_d is the dissociation constant of fura-2 (225 nM) and S_{f2}/S_{b2} is the ratio of intensities at the denominator wavelength for the free and bound dye. This factor corrects for wavelength biasing due to instrumental artifacts.

Statistics. Statistical significance was assessed with unpaired *t* tests, using the Statworks 1.1 software.

Results

Several lines of CHL fibroblasts expressing cloned rat GLP-1 receptors (between 50,000 and 250,000 binding sites/cell) were obtained. All of the cell lines analyzed produced cAMP when stimulated with GLP-1 (data not shown). For our present studies we used clone 5, which expressed 50,000–100,000 GLP-1 binding sites/cell, with a K_d of 160 pM, an affinity that is very similar to that measured in INS-1 cells (120 pM) (7). We also used different insulinoma cell lines. These cells may represent different states of dedifferentiation of natural β cells. Therefore, to minimize the possibility that the observed coupling could result from the particular state of transformation of one insulinoma, we tested the coupling of the GLP-1 receptor in different cell lines.

cAMP production. Fig. 1A shows that cAMP accumulation in three insulinoma cell lines and in clone 5 increased dose-dependently in response to added GLP-1. The dose-response curves were nearly superimposible for the four cell lines tested, with half-maximal production of cAMP being reached at about 0.5 nM GLP-1. Similar experiments with COS cells transformed with the GLP-1 receptor cDNA gave half-maximal production at 0.3 nM GLP-1 (data not shown). This indicates that the cloned rat GLP-1 receptor is able to activate adenylyl cyclase as do receptors endogenously expressed in insulinomas.

We further demonstrated that coupling of the GLP-1 receptor to adenylyl cyclase could be observed with crude membrane preparations obtained from clone 5 cells. In the experiment described in Fig. 1B, GLP-1 elicited a dose-dependent increase in cAMP production in clone 5 membranes. The EC_{50} for cAMP production was, however, obtained at higher concentrations of GLP-1, which may indicate a lower coupling efficiency in this membrane preparation, compared with intact cells. Such differences in the EC_{50} for cAMP production between intact cells and membrane preparations have been observed for several G protein-coupled receptors (28–30).

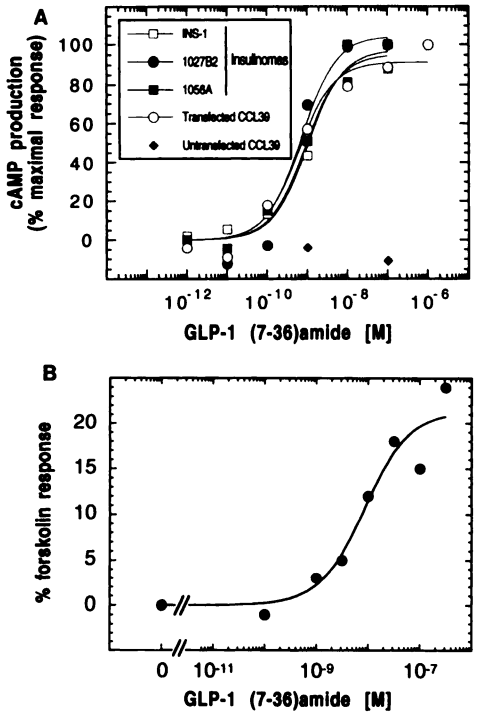

Fig. 1. GLP-1 stimulation of cAMP formation in insulinomas and GLP-1 receptor-transfected CHL fibroblasts. A, [^{3}H]Adenine-loaded cells were stimulated for 10 min at 37° with increasing GLP-1 concentrations. The amount of accumulated intracellular [^{3}H]cAMP was then determined (in triplicate) as described in Materials and Methods. Results are expressed as the percentage of the maximal response, with basal levels subtracted, to allow comparison between the different cell lines. cAMP production of untransfected fibroblasts was normalized to the maximal response of transfected fibroblasts (clone 5). Basal levels (no agonist) were 186 ± 16 (INS-1), 267 ± 20 (1027B2), 122 ± 15 (1056A), 146 ± 12 (transfected CHL fibroblasts, clone 5), and 90 ± 9 cpm (untransfected CHL fibroblasts). Maximal [^{3}H]cAMP accumulation was 2316 ± 594 (INS-1), 514 ± 63 (1027B2), 619 ± 120 (1056A), and 434 ± 54 cpm (transfected CHL fibroblasts, clone 5). B, Adenylyl cyclase activity of crude membranes of clone 5 incubated with the indicated GLP-1 concentrations or with 100 μM forskolin was assessed as described in Materials and Methods. Results were expressed as percentage of the forskolin response after subtraction of the basal cAMP production. Production of [^{32}P]cAMP in the presence of 100 μM forskolin was 5777 ± 462 cpm. Basal [^{32}P]cAMP production (in the absence of agonist) was 1265 ± 36 cpm.

When the rate of cAMP production in clone 5 cells was measured in response to maximally stimulating concentrations of GLP-1 (100 nM) in the presence of the phosphodiesterase inhibitor IBMX, accumulation of cAMP increased linearly over the first 30–60 sec and reached a plateau value at about 2–3 min (Fig. 2, A and C). In the absence of IBMX a qualitatively similar pattern was observed, with a plateau at a lower cAMP level (Fig. 2A). The same pattern of cAMP accumulation in the presence of IBMX was observed in the INS-1 insulinoma (Fig. 2B). However, no detectable cAMP accumulation in the absence of the phosphodiesterase inhibitor could be observed in this case (Fig. 2B). Lack of cAMP accumulation in GLP-1-stimulated INS-1 cells in the absence of IBMX could be due to the relatively small number of receptors expressed by these cells (2000 binding sites/cell), compared with clone 5 (50,000–100,000 binding sites/cell). Alternatively, phosphodiesterase activities may be controlled differently in the two cell lines.

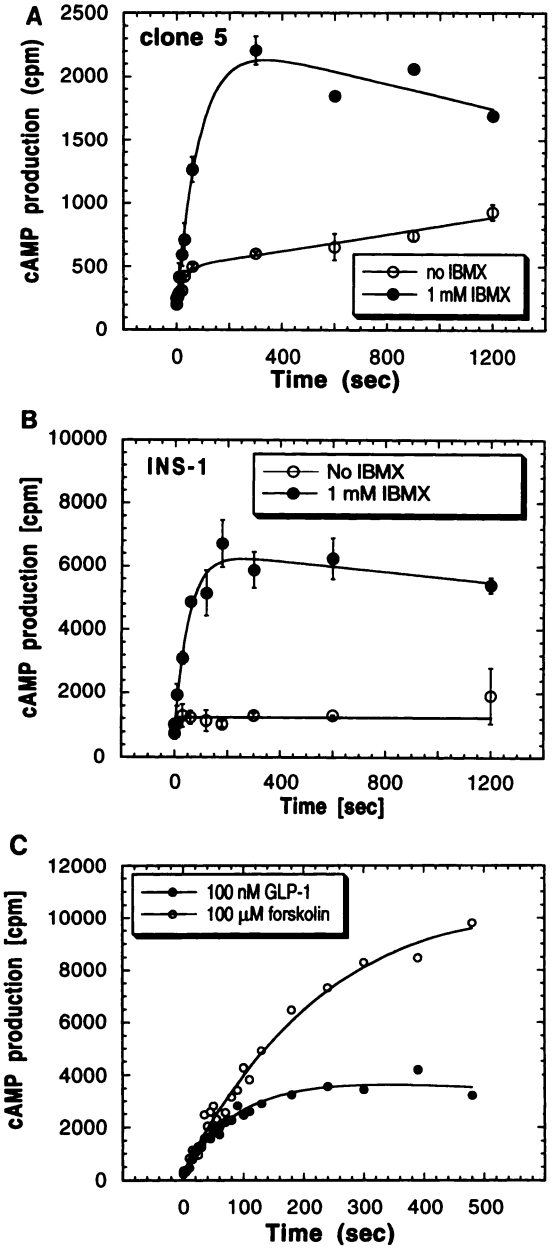

Fig. 2. Kinetics of cAMP production by stimulated GLP-1 receptors. [^{3}H] Adenine-loaded clone 5 and INS-1 cells were stimulated for the indicated periods of time at 37° with different agonists, in the presence or in the absence of 1 mM IBMX. Total [^{3}H]cAMP accumulation was determined (in duplicate) as described in Materials and Methods. A and B, Kinetics of cAMP production by clone 5 cells (A) and INS-1 cells (B) stimulated with 100 nM GLP-1. C, Kinetics of cAMP production by clone 5 cells stimulated with 100 nM GLP-1 or with 100 μM forskolin, in both cases in the presence of 1 mM IBMX. Basal levels in the absence of agonist were 192 ± 70 (0-min incubation, no IBMX), 299 ± 98 (20-min incubation, no IBMX), 203 ± 43 (0-min incubation, 1 mM IBMX), and 552 ± 256 cpm (20-min incubation, 1 mM IBMX) (A) and 1029 ± 207 (0-min incubation, no IBMX), 1270 ± 47 (20-min incubation, no IBMX), 728 ± 143 (0-min incubation, 1 mM IBMX), and 1968 ± 385 cpm (20-min incubation, 1 mM IBMX) (B). Production of cAMP by INS-1 cells (B) after 20-min stimulation with 100 μM forskolin was 3310 ± 38 (no IBMX) and 10,162 ± 56 cpm (1 mM IBMX).

The rates of cAMP accumulation induced by GLP-1 and forskolin (a direct activator of adenylyl cyclase) in clone 5 cells were apparently identical during the first 30–45 sec of stimulation (Fig. 2C). Only after 45 sec did the two curves separate, with the GLP-1 response starting to plateau after about 2–3 min. This was not due to exhaustion of the intracellular tritiated ATP pool, because forskolin-induced cAMP production continued to rise with time (Fig. 2C). A similar effect was observed in INS-1 cells (see the legend to Fig. 2). Thus, these data suggest that the decreased cAMP response after prolonged exposure to GLP-1 might result from receptor desensitization.

GLP-1 receptor coupling to adenylyl cyclase in the HIT insulinoma cell line has been suggested to be glucose dependent (31). We therefore tested the cAMP response of clone 5 and INS-1 cells after stimulation with GLP-1 in the absence and presence of different glucose concentrations (Table 1). Before stimulation, cells were preincubated in the absence of glucose, either in a saline solution as described (31) or in a glucose-free culture medium. After this preincubation period, cells were stimulated with GLP-1 or forskolin in the absence or presence of 2.8 or 16.7 mM glucose. In contrast to the data reported for HIT cells, the presence of glucose at different concentrations during the stimulation period did not significantly change the production of cAMP in response to either GLP-1 or forskolin stimulation (Table 1). Thus, the stimulation of cAMP production by both cell types was already maximal in the absence of glucose.

Inositol phosphate production. Inositol phosphate production in response to GLP-1 was tested in insulinoma cell lines and in clone 5. Under no conditions could we measure an increase of inositol phosphate production (Fig. 3). These cell lines have, however, the potential to produce inositol phosphates in response to carbachol (acting on endogenous muscarinic cholinergic receptors expressed by insulinoma cells) or to thrombin (acting on endogenous receptors expressed by CHL cells) (Fig. 3).

Inositol phosphate production in response to GLP-1 has been reported in transiently transfected COS cells (13). We thus cotransfected COS cells with the GLP-1 receptor and the α_{1B}-adrenergic receptor. The latter is known to be physiologically coupled to inositol phosphate production (24) and served as an internal control in our experiment. In GLP-1 receptor-transfected COS cells, about 10×10^6 binding sites were expressed at the cell surface. Under such conditions, GLP-1 stimulated cAMP production as strongly as did isoproterenol acting on endogenous β_2-adrenergic receptors (Fig. 4, *left*) or as did 100 μM forskolin (data not shown). However, in contrast to the effect of norepinephrine acting at the α_{1B}-adrenergic receptor

TABLE 1

cAMP production by stimulated GLP-1 receptors is not glucose dependent

Transfected CHL fibroblasts (clone 5) and INS-1 insulinoma cells were loaded with tritiated adenine and incubated for 1 hr at 37° in HBS without glucose (glucose-free saline solution) or in RPMI 1640 medium without glucose. IBMX (1 mM) was then added and the cells were stimulated with either 10 nM GLP-1 or 100 μM forskolin, in the presence of 0, 2.8, or 16.7 mM glucose. The cAMP production was determined in quadruplicate as described in Materials and Methods. Increasing glucose concentrations did not significantly change the forskolin-induced cAMP production.

Glucose concentration during stimulation	GLP-1-induced response		
	Glucose-free saline solution		Glucose-free RPMI 1640, INS-1
	Clone 5	INS-1	
mM	*% of forskolin response*		
0	40 ± 4	30 ± 3	28 ± 4
2.8	Not done	27 ± 3	32 ± 8
16.7	40 ± 5	29 ± 5	36 ± 5

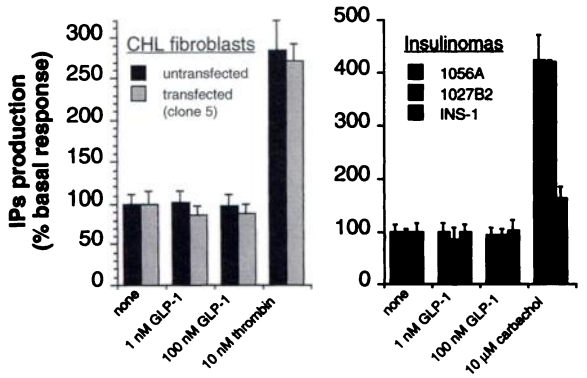

Fig. 3. Lack of stimulation by GLP-1 of inositol phosphate (*IPs*) production. [^{3}H]Inositol-loaded cells were stimulated for 20 min at 37° with the indicated agonists. Intracellular [^{3}H]inositol phosphate accumulation was then determined (in triplicate) as described in Materials and Methods. Results are expressed as the percentage of the basal response. Basal levels (no agonist) were 1177 ± 69 (untransfected CHL fibroblasts), 1267 ± 92 (transfected CHL fibroblasts, clone 5), 652 ± 57 (1056A), 1204 ± 57 (1027B2), and 11,625 ± 1,464 cpm (INS-1).

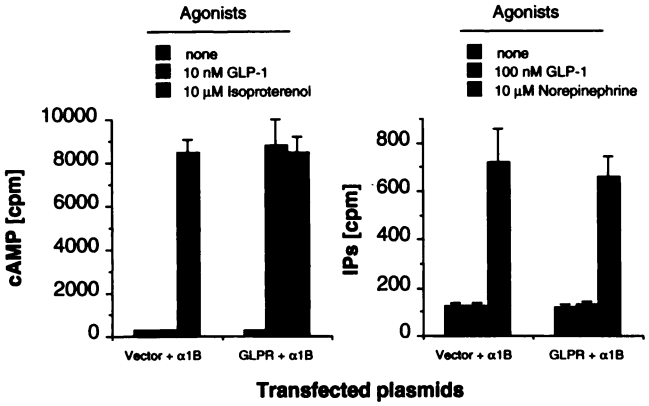

Fig. 4. Second messengers generated by GLP-1 in GLP-1 receptor-transfected COS cells. COS cells were transiently transfected with plasmids pcDNA-1 and pBC12BI-α1B (*Vector + α1B*) or with plasmids pGLPR-1 and pBC12BI-α1B (*GLPR + α1B*), as described in Materials and Methods. Plasmids pGLPR-1 and pBC12BI-α1B contain cDNAs encoding the rat GLP-1 receptor (7) and the hamster α_{1B}-adrenergic receptor (24), respectively. Plasmid pcDNA-1 is the vector used to construct plasmid pGLPR-1. Transfected COS cells were loaded either with tritiated adenine or with tritiated inositol, as described in Materials and Methods. They were then stimulated with the indicated agonists. Stimulation was for 8 min (cAMP measurements) or 30 min (inositol phosphate measurements) at 37°. The amounts of accumulated intracellular tritiated cAMP and tritiated inositol phosphates (*IPs*) were determined as described in Materials and Methods.

level, GLP-1 could generate only a very small inositol phosphate production (7.5% above basal, 1.7% of the norepinephrine response) (Fig. 4, *right*). Similar results were obtained when the GLP-1 receptor was transfected alone (data not shown). This latter result excluded the possibility of any competition between this receptor and the α_{1B}-adrenergic receptor for any limiting component in the inositol phosphate generation process. The small inositol phosphate production induced by GLP-1 in COS cells could not be reproducibly observed. In four additional experiments, the GLP-1-induced inositol phosphate accumulation was 19.6% above basal levels in two experiments and below the basal levels in the other two. We thus conclude that the GLP-1 receptor is not coupled or only weakly coupled to phospholipase C in COS cells.

Intracellular calcium mobilization. The possibility that activation of the GLP-1 receptor could lead to intracellular calcium mobilization was assessed using the calcium probes fura-2 (Fig. 5) and fluo-3 (data not shown). No intracellular calcium concentration increase was observed when suspensions of four different insulinoma cells and clone 5 were stimulated with GLP-1, although all of the cells responded well to control stimuli (Fig. 5). The cell detachment process was apparently not responsible for the absence of calcium response after GLP-1 addition. Indeed, trypsin detachment of the cells did not affect the ability of clone 5 to generate cAMP responses to GLP-1 (data not shown). Furthermore, no increase in intracellular calcium concentration could be recorded when calcium measurements were performed on adherent clone 5 cells with the indicator fluo-3 under the confocal microscope (data not shown).

It was reported that COS cells transiently transfected with the GLP-1 receptor displayed an increased intracellular calcium concentration after exposure to GLP-1 (13). COS cells were thus cotransfected with GLP-1 and α_{1B}-adrenergic receptor cDNAs. An increase in the intracellular calcium concentration after GLP-1 stimulation could indeed be observed (Fig. 6). When compared with the response generated by norepinephrine acting at the α_{1B}-adrenergic receptor level, however, the GLP-1-induced calcium response was much smaller. Similar results were obtained when GLP-1 receptor cDNA and α_{1B}-adrenergic receptor cDNA were transfected separately (Fig. 6). Taken together, our results show that activation of the GLP-1 receptor leads to a weak rise in the intracellular calcium concentration only when the receptor is overexpressed in transiently transfected COS cells.

Discussion

In the present report we characterized the coupling of the cloned GLP-1 receptor, expressed in COS cells and fibroblasts, to the adenylyl cyclase and phospholipase C pathways. We also

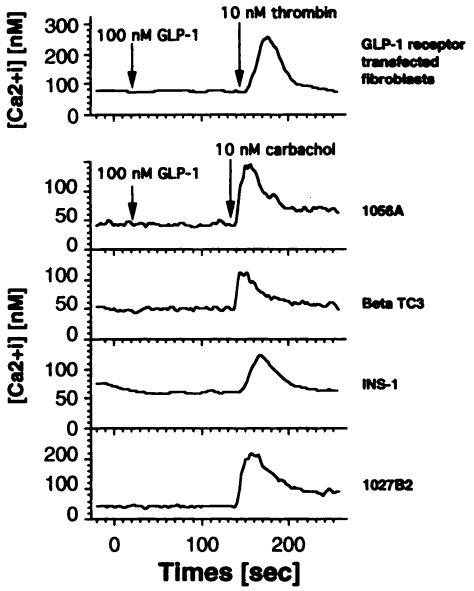

Fig. 5. Lack of induction of free cytosolic calcium increase by GLP-1 in insulinomas and GLP-1 receptor-transfected CHL fibroblasts. Cells loaded with the calcium indicator fura-2 were stimulated at the indicated time with the indicated agonists. Increases in the intracellular calcium concentration were determined as described in Materials and Methods.

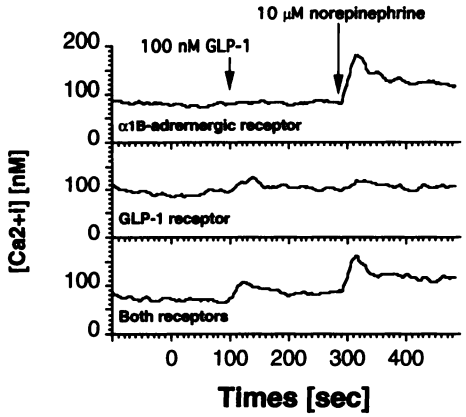

Fig. 6. Calcium mobilization by GLP-1 in GLP-1 receptor-transfected COS cells. COS cells were transiently transfected with plasmids encoding the rat GLP-1 receptor and the hamster α_{1B}-adrenergic receptor as indicated (see the legend to Fig. 4). They were then processed as described in the legend to Fig. 5.

compared the signaling properties of the cloned receptor with those of the endogenous receptor expressed in different insulinoma cell lines.

Coupling of the GLP-1 receptor to the production of cAMP was observed in three different cell types, i.e., transiently transfected COS cells, stably transfected CHL fibroblasts, and insulinoma cell lines. The accumulation of cAMP in response to GLP-1 proceeded with a similar dose dependence in the three different cell types, although the number of receptors expressed per cell varied widely among the cells studied (about 2,000 for INS-1 cells, 50,000–100,000 for fibroblasts, and close to 10×10^6 for COS cells). Production of cAMP stimulated by GLP-1 receptors is most likely due to direct coupling of the receptor to G_s and adenylyl cyclase, because adenylyl cyclase is activated by GLP-1 on clone 5 membrane preparations. The initial rate of cAMP accumulation in response to GLP-1, as well as to forskolin, in the presence of the phosphodiesterase inhibitor IBMX was similar in fibroblasts and insulinomas. However, in contrast to the forskolin-mediated response, the receptor-dependent cAMP production rapidly reached a plateau value within 2–3 min. These observations can be interpreted as rapid desensitization of the receptor after agonist stimulation.

Lu and collaborators previously reported that cAMP production by insulinoma cells in response to GLP-1 was dependent on glucose (31). In contrast, our results showed that glucose deprivation did not modify GLP-1-induced cAMP production in either transfected fibroblasts or INS-1 cells, indicating that glucose is not required for receptor coupling to adenylyl cyclase. The glucose-dependent coupling of GLP-1 receptors to cAMP production observed by these authors may be due to the specific cell line they studied but is not an intrinsic characteristic of the function of the receptor.

Several G protein-coupled receptors are coupled to more than one intracellular signaling pathway (17, 18). Is this the case for the GLP-1 receptor? In a recent report, Wheeler *et al.* (13) presented evidence for GLP-1 receptor coupling to both adenylyl cyclase and phospholipase C pathways in transfected COS cells. We thus investigated whether GLP-1-mediated activation of the phospholipase pathway could be generalized to other cell types. Inositol phosphate levels and changes in intracellular calcium concentrations were measured in different cell lines in response to GLP-1 and were compared with the effects generated by activation of the α_{1B}-adrenergic receptor, thrombin receptor, and muscarinic cholinergic receptor. In the present study, a rise in intracellular calcium concentrations after GLP-1 binding to its receptor could be observed only in transfected COS cells. This signal was, however, much smaller than that generated by activation of the α_{1B}-adrenergic receptor, a receptor known to be physiologically coupled to phospholipase C. Moreover, we were unable to detect clear and reproducible inositol phosphate production in transfected COS cells in response to GLP-1. The discrepancy between our study and that of Wheeler *et al.* (13) is not clearly understood but may be due to the different techniques used to detect inositol phosphate formation. When transfected fibroblasts or the different insulinoma cell lines were studied, no coupling to inositol phosphate production or intracellular calcium mobilization could be observed.

Therefore, coupling of the GLP-1 receptor to phospholipase C is most probably not physiologically important in the potentiation of insulin secretion by GLP-1, because (i) the coupling observed in COS cells is rather weak, compared with the effect of norepinephrine acting on transfected α_{1B}-adrenergic receptors and (ii) no such coupling could be observed in transfected fibroblasts or in the different insulinoma cell lines tested. Moreover, it has been shown that phosphoinositide hydrolysis is not involved in GLP-1-induced potentiation of insulin secretion in rat islet cells (32). Increases in the intracellular calcium concentration in COS cells may result from the very high levels of receptor expression in these cells or could be due to a cell-specific activation of phospholipase C not present in β cells. Thus, our data suggest that, in β cells, interaction between glucose signaling and activated GLP-1 receptors is mediated only by activation of adenylyl cyclase, with the consequent production of cAMP and activation of cAMP-dependent protein kinase.

Glucose-induced insulin secretion by β cells requires glucose uptake through the GLUT-2 glucose transporter isoform and glucose metabolism. As a result of glucose metabolism there is a rise in the cytoplasmic ATP/ADP ratio, which induces the closure of an ATP-dependent potassium channel. This block in potassium efflux depolarizes the plasma membrane, which, in turn, leads to the opening of a voltage-gated calcium channel. The consequent increase in intracellular calcium concentration triggers the exocytosis of insulin-containing secretory granules (19, 33, 34). The most likely hypothesis to explain GLP-1 modulation of this cascade of events is that activated protein kinase A phosphorylates components of this signaling system (11). Likely targets for the kinase activity are the ATP-dependent potassium channel (15), the voltage-dependent calcium channels (35), the glucose transporter GLUT-2, or elements of the secretory machinery itself (16).

Finally, our data have shown that GLP-1 receptor-expressing CHL fibroblasts have very similar signaling properties, compared with insulinoma cell lines. These cells will thus be of great help for further biochemical studies. Our goal is now to understand in molecular terms how the receptor couples to G proteins and how the receptor is desensitized.

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