ABSTRACT

The insulinotropic hormone glucagon-like peptide-1 (7-36)-amide (GLP-1) has potent effects on glucose-dependent insulin secretion, insulin gene expression, and pancreatic islet cell formation and is presently in clinical trials as a therapy for type 2 diabetes mellitus. We report on the effects of GLP-1 and two of its long-acting analogs, exendin-4 and exendin-4 WOT, on neuronal proliferation and differentiation, and on the metabolism of two neuronal proteins in the rat pheochromocytoma (PC12) cell line, which has been shown to express the GLP-1 receptor. We observed that GLP-1 and exendin-4 induced neurite outgrowth in a manner similar to nerve growth factor (NGF), which was reversed by coincubation with the selective GLP-1 receptor antagonist exendin (9-39). Furthermore, exendin-4 could promote NGF-initiated differentiation and may rescue degenerating cells after NGF-mediated withdrawal. These effects were induced in the absence of cellular dysfunction and toxicity as quantitatively measured by 3-(4,5-dimethyl-2-yl)-2,5-diphenyl tetrazolium bromide and lactate dehydrogenase assays, respectively. Our findings suggest that such peptides may be used in reversing or halting the neurodegenerative process observed in neurodegenerative diseases, such as the peripheral neuropathy associated with type 2 diabetes mellitus and Alzheimer’s and Parkinson’s diseases. Due to its novel twin action, GLP-1 and exendin-4 have therapeutic potential for the treatment of diabetic peripheral neuropathy and these central nervous system disorders.

Diabetes is now the major cause of peripheral neuropathy in the United States. Twenty to thirty percent of all diabetic subjects eventually develop peripheral neuropathy. In addition to type 2 diabetes, central nervous system (CNS) disorders such as Alzheimer’s disease also display neuropathy, including a characteristic synaptic loss and neurodegeneration. There are reports of increased risk of Alzheimer’s disease with heart disease, stroke, hypertension, and diabetes mellitus (Ott et al., 1999). GLP-1 has been identified as an endogenous insulinotropic peptide, which is synthesized and secreted from the L-cells of the gastrointestinal tract in response to food (Gutniak et al., 1992). When given exogenously it lowers blood glucose levels in type 2 diabetic and nondiabetic patients, primarily by stimulating insulin secretion. Despite its promise, the use of GLP-1 as a therapeutic agent for the treatment of type 2 diabetes mellitus is critically undermined by its susceptibility to proteolytic degradation (Kieffer et al., 1995); the half-life of the compound is only 1.5 min in rodents (Greig et al., 1999).

Exendin-4, an analog of GLP-1, binds to the known GLP-1 receptor in pancreatic β cells (Goke et al., 1993; Thorens et al., 1993). Exendin-4 has several advantages over GLP-1; it has a higher potency than GLP-1, its half-life is approximately 120 min, and it maintains higher plasma levels of insulin over a longer time duration than GLP-1 (Wang et al., 1997; Ryan et al., 1998; Greig et al., 1999). Derived from the saliva of the Gila monster lizard, it has 53% amino acid homology with GLP-1 (Pohl and Wank, 1998). In addition to insulinotropic action, GLP-1 and analogs increase pancreatic islet cell mass (Zhou et al., 1999; Perfetti et al., 2000) and

ABBREVIATIONS: CNS, central nervous system; GLP-1, glucagon-like peptide-1 (7-36)-amide; NGF, nerve growth factor; FBS, fetal bovine serum; PKA, protein kinase A; RT-PCR, reverse transcriptase-polymerase chain reaction; BrdU, 5’-bromo-2’-deoxy-uridine; MTT, 3-(4,5-cimethy1thiazol-2-yl)-2,5-diphenyl tetrazolium bromide; LDH, lactate dehydrogenase; MTS, 3-(4,5-dimethyl-2-yl)-5-(3 carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; ERK, extracellular signal-regulated kinase; MAP, mitogen-activated protein; PI3-kinase, phosphatidylinositol 3-kinase.
insulin content (Hussain and Habener, 2000; Perfetti et al., 2000).

We have synthesized a number of novel GLP-1 analogs, which bind at the GLP-1 receptor and combine some of the best features of GLP-1 and exendin-4. Although predominantly located in pancreatic islets, recent reports have shown GLP-1 receptor expression in lung and brain (Kanse et al., 1988; Thoren, 1992; Campos et al., 1994). A CNS role for GLP-1 has been established from considerable experimental data showing that intracerebroventricular infusion of GLP-1 inhibits feeding in rats (Turton et al., 1996). Similarly, peripheral administration of exendin-4 also reduces food intake and body weight (Szayna et al., 2000) in rats. GLP-1 receptor knockout mice, although exhibiting abnormal glucose homeostasis due to defective glucose-induced insulin secretion, do not respond to GLP-1 administration and exhibit no GLP-1 receptor binding sites in either the pancreas or the CNS (Scrochi et al., 1996). Surprisingly, such mice are lean, eat normally, and do not become obese, although there appears to be some disruption of neuroendocrine responses to stress as a result of impaired CNS-pituitary-adrenal function (MacLusky et al., 2000). In the brain, glucagon-like peptides are synthesized in the caudal brainstem and in the hypothalamus (Campos et al., 1994) with the highest density of receptors appearing largely confined to circumventricular areas. However, GLP-1 receptor presence has also been demonstrated on glial cells in the rodent brain after mechanical injury (Chowen et al., 1999).

Taken together, these studies suggest a possible neurotrophic role for glucagon-like peptides within the central nervous system. In light of this, and as a consequence of our interest in neurodegeneration, we have assessed the action of GLP-1 and analogs in PC12 cells. This cell system has been widely used as a model system for studying the mechanisms associated with neuronal differentiation. When treated with NGF, these cells differentiate into sympathetic-like neurons (Greene and Tischler, 1976). NGF is a well studied, large, basic 118 amino acid protein that acts as a trophic factor for neuronal differentiation. When treated with NGF, these cells differentiate into sympathetic-like neurons (Chowen et al., 1999).

Here, we examine in detail the trophic effects of GLP-1 and related analogs clearly have important implications for the treatment of both central and peripheral neurodegenerative disorders, such as the neuropathy associated with type 2 diabetes mellitus, and Alzheimer’s and Parkinson’s diseases.

**Experimental Procedures**

**Materials**

Cell culture media and sera were obtained from Mediatech (Herdon, VA). NGF (7S-subunit) was purchased from Promega (Madison, WI). GLP-1 and exendin (9-39) were obtained from Bachem (Torrance, CA). Exendin-4 and its analog exendin-4 WOT were synthesized and assessed to be >95% pure by high-performance liquid chromatography analysis. Their amino acid sequences are shown in Fig. 1. All other chemicals were of high purity and obtained from Sigma Chemical (St. Louis, MO), unless otherwise stated.

### GLP-1 as a Novel Neurotrophic Factor in PC12 Cells

**Cell Culture and Drug Treatment of Pheochromocytoma (PC12) Cells**

PC12 cells were cultured in RPMI 1640 supplemented with 10% horse serum and 5% fetal bovine serum (FBS) as described (Lahiri et al., 2000). RIN 1046-38 cells (a clonal rat insulinoma cell line obtained from Dr. S. A. Clark, Bio Hybrid Technology, Shrewsbury, MA) were grown in medium 199 containing 12 mM glucose and supplemented with 5% FBS as described (Montrose-Rafizadeh et al., 1994). Cells were seeded at approximately 2.0 x 10^6 cells/60-mm dish. PC12 cells were grown on cultureware coated in rat-tail collagen (Roche Molecular Biochemicals, Indianapolis, IN).

Treatments in triplicate begun 24 h after seeding, once cells were well attached. The medium was aspirated, and 3 ml of fresh low serum media containing 0.5% FBS with the appropriate compound(s) was added.

PC12 cells were treated with NGF (10, 30, 50, and 100 ng/ml), with exendin-4 (0.1, 1, and 10 μg/ml), with exendin-4 WOT (0.1 and 1 μg/ml), and with GLP-1 (3.3, 33, and 330 μg/ml). In addition, NGF and exendin-4 were added simultaneously in combination at 10 and 100 ng/ml, and at 30 and 100 ng/ml, respectively.

To examine the mechanism of action of GLP-1 and exendin-4, 48-h drug treatments were carried out with the GLP-1 antagonist exendin (9-39) (0.1 and 9 μM for GLP-1 and exendin-4 antagonist, respectively); with the PI3 kinase inhibitor LY294002 (40 μM); with the mitogen-activated protein kinase inhibitor PD98059 (50 μM); and with the PKA inhibitor H89 (20 μM). Cells were seeded onto 60-mm dishes at approximately 1 x 10^6 cells/ml and treated with either 10 nM GLP-1 (33 μg/ml) or 0.3 μM exendin-4 (1 μg/ml) and with each of the aforementioned compounds. NGF at 50 ng/ml and 20 μM forskolin (PKA activator) were used as positive controls in these treatments.

### Demonstration of GLP-1 Receptor Presence in PC12 Cells

**Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)**

Total RNA was isolated from PC12 cells by using the method of Chomczynski and Sacchi (1987). RT-PCR was undertaken with 2.5 μg RNA, in a volume of 50 μl, containing 1 to 1 x 10^6 cells/ml and treated with either 10 nM GLP-1 (33 μg/ml) or 0.3 μM exendin-4 (1 μg/ml) and with each of the aforementioned compounds. NGF at 50 ng/ml and 20 μM forskolin (PKA activator) were used as positive controls in these treatments.

### cAMP Determination

Cyclic AMP was measured according to the method of Montrose-Rafizadeh et al. (1997). Triplicate PC12 cell cultures were treated with 33 μg/ml GLP-1 and harvested at 5-min intervals after the onset of drug treatment for a total period of 30 min. Cells harvested

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**Fig. 1.** Amino acid sequences for GLP-1, exendin-4, exendin (9-39), and exendin-4 WOT peptides.
at the start of drug treatment (0 min) were used for baseline levels of cAMP.

Cell Turnover in PC12 Cells Determined by Incorporation of BrdU, 5′-Bromo-2′-deoxy-uridine (BrdU)

PC12 cells were cultured for 3 days in the presence of vehicle, 33 μg/ml GLP-1, or 50 ng/ml NGF. To label cellular DNA 10 μM BrdU was added to the culture medium for 6 h before fixing in 4% paraformaldehyde. The remainder of the method was followed according to the proliferation kit (Roche Molecular Biochemicals). Proliferating cells (those that were undergoing DNA replication at the time of BrdU labeling) exhibited dark-staining nuclei with the chromagen reaction. BrdU incorporation was quantitated on days 1, 2, and 3 of treatment. Three dishes for each treatment condition were counted and expressed as the percentage of labeled cells relative to the total number of cells.

Quantification of Exendin-4-Mediated Neurite Outgrowth

PC12 cells were cultured in low serum media on 60-mm dishes as described above, for 4 days. During this time neurite outgrowth was quantified daily by using phase contrast microscopy. Five random fields of cells were evaluated per dish and the proportion of neurite-bearing cells was determined. Approximately 100 cells per field were scored for neurites equal to or greater in length than that of the cell body. A cell was only scored once, although it may have had more than one process per cell.

Effect of Exendin-4 on NGF-Mediated Cell Death

PC12 cells were grown on collagen-coated dishes as described above, in regular media in the presence of a vehicle, NGF (50 ng/ml), or exendin-4 (1 or 5 μg/ml). Cells were harvested after 4 or 7 days, and subsequently allowed to rejuvenate in regular media for an additional 3 days. On the final day, cells were harvested and an MTT assay was performed to determine the proportion of viable cells.

In a second series of experiments (prevention) cells were cultured in the presence of NGF and exendin-4 for 4 or 7 days. Cells were harvested and allowed to rejuvenate as described above.

In a third series of experiments (rescue) cells were cultured in the presence of NGF for 4 days. Exendin-4 was added to the media for an additional 3 days. Cells were harvested on day 7 and allowed to rejuvenate as described above.

In a fourth series of experiments (rescue), cells were grown in the presence of NGF. On day 4, exendin-4 was added for an additional 3 days. Cells were harvested on day 7 and allowed to rejuvenate as described above.

Cells were counted in each plate by the trypan blue exclusion method and MTT assays were performed on days 4 and 7. Total lactate dehydrogenase (LDH) and secreted LDH in the media were measured in all plates and the percentage of LDH release was calculated (see below).

Preparation of Cell Lysates

Cells were collected gently by trituration of the media and centrifuged at 800g for 10 min. After washing in phosphate-buffered saline, pH 7.4, the cell pellet was suspended in lysis buffer containing 50 mM Tris-HCl, pH 7.4, 2% SDS, 0.174 mg/ml phenylmethysulfon-yl fluoride, 1 μg/ml each of aproatin, leupeptin, and peptatin A, and 4 μl of a mixture of 45.98 mg/ml sodium vanadate and 10.5 mg/ml sodium fluoride. The suspended cells were trituated and centrifuged at 14,000g for 15 min. The proteins of the supernatant solution (cell lysate) were measured (Bradford, 1976) and analyzed by immunoblotting.

LDH Assay

Conditioned media and cell pellet samples collected at different time intervals after drug treatments were subjected to a sensitive LDH assay by using a Sigma kit. The LDH assay provides a measure of the number of cells via total cytoplasmic LDH or by membrane integrity as a function of the amount of cytoplasmic LDH released into the medium. The measurement of released LDH is based on the reduction of NAD by the action of LDH. The resulting reduced NAD (NADH) is used in the stoichiometric conversion of a tetrazolium dye. The final colored compound is measured colorimetrically.

MTT Assay

We have used the CellTiter 96 Aqueous One Solution Cell Proliferation Assay Reagent from Promega. This colorimetric procedure for determination of the number of viable cells in proliferation or cytotoxicity assays is a slight modification from the MTT assay. The reagent contains a novel tetrazolium compound [3-(4,5-dimethyl-2-yl)-5-(3 carboxymethoxyphenyl)-2(4-sulfophenyl)-2H-tetrazolium, inner salt; MTS] and an electron coupling reagent (phenazine ethosulfate). Phenazine ethosulfate has enhanced chemical stability, which allows combination with MTS to form a stable solution. The MTS tetrazolium compound (Owners’ reagent) is biorediayed by cells into a colored formazan product, which is soluble in tissue culture medium. This conversion is presumably accomplished by NADPH or NADH produced by dehydrogenase enzymes in metabolically active cells. Assays are performed by the addition of a small amount of the reagent directly to cultured wells, incubation for 1 to 4 h and subsequent absorbance at 490 nm with a 96-well plate reader. The quantity of formazan product, as measured by the amount of 490-nm absorbance, is directly proportional to the number of living cells in the culture.

Protein Analysis by Western Blotting

Western blot analysis was performed on 10 μg of protein from each cell lysate and conditioned media sample by using 10% Tris-glycine gels containing 2.6% bis-acrylamide (Invitrogen, Carlsbad, CA). Proteins were blotted onto polyvinylidene difluoride paper. Transferred proteins were visualized by staining the membrane with 0.1% Ponceau S solution in 5% acetic acid (Sigma Chemical) and the immunoreactive bands were detected using specific antibodies as described below.

Immunodetection

The synaptophysin monoclonal antibody (Oncogene Research Products, San Diego, CA), which stains neurosecretory vesicles of PC12 cells, was used as a probe to examine the extent of differentiation in response to the various treatments. To investigate the role of the transcription factor Beta-2/NeuroD in GLP-1-mediated differentiation in PC12 cells, cell lysates were probed with the NeuroD polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA). To establish whether any differences in the profile of immunoreactive bands were due to unequal loading of protein into the lanes of the gel, cell lysate blots were probed with the polyclonal β-actin antibody (Santa Cruz Biotechnology).

The membranes were blocked with 20 mM Tris, 500 mM NaCl, pH 7.4, 1% (w/v) casein (Bio-Rad, Hercules, CA) at 37°C for 1 h. Primary antibody was diluted in block and incubated with the proteins overnight at 4°C. The membrane was vigorously washed with 20 mM Tris, pH 7.4, 150 mM NaCl, and 0.05% Tween 20. The peroxidase-linked secondary antibody in block was incubated with the membrane for 1 h at room temperature. Peroxidase-linked anti-mouse IgM (Chemicon International, Temecula, CA) and anti-goat IgG (Santa Cruz Biotechnology) were used as the secondary antibodies against synaptophysin and NeuroD, respectively. Excess antibody was washed off with 20 mM Tris, pH 7.4, 150 mM NaCl, and 0.05% Tween 20 before incubation in ECL Plus (Amersham Biosciences, Inc., Piscataway, NJ) for 5 min. The membrane was subsequently exposed to photographic film. Visualization of the β-actin signal used the avidin-biotin peroxidase method with subsequent development in diaminobenzidine dihydrochloride after incubation in the biotinylated anti-rabbit IgG secondary antibody from the Vectord Elite ABC.
kit (Vector Laboratories, Burlingame, CA). Densitometric quantification of the protein bands was performed using Molecular Analyst software (Bio-Rad).

**Data Analysis**

Results are expressed as mean ± S.E.M. (where S.E.M. is standard error of the difference between the means). Analysis of variance was carried out using SPSS version VII (SPSS Inc., Chicago, IL), where p < 0.05 was considered statistically significant. Following significant main effects, planned comparisons were made using Tukey’s honestly significant difference test.

**Results**

**GLP-1 Receptor Presence and Activity.** RT-PCR was performed, as a more sensitive assay for GLP-1 receptor mRNA. Rat insulinoma cells (RIN cells) were used as a positive control. RT-PCR products of the expected size for the GLP-1 receptor with the primers used were obtained. Clear bands at 928 base pairs in rat islet mRNA and PC12 cell mRNA (Fig. 2A) confirmed the presence of the GLP-1 receptor on PC12 cells.

Activation of the GLP-1 receptor has been shown to stimulate adenylyl cyclase, leading to an increase in intracellular cAMP. Cyclic AMP was assayed over 30 min after treatment of PC12 cells with 33 µg/ml GLP-1 (Fig. 2B). There was a maximal 1200-fold increase in cAMP levels within 15 min of stimulation, which returned to near baseline within 30 min.

Taken together these findings demonstrate the presence and activity of the GLP-1 receptor on PC12 cells.

**GLP-1 Does Not Affect Proliferation of PC12 Cells.** We considered the possibility that GLP-1 might affect the proliferation of PC12 cells in culture. To clarify this issue, we assessed cell proliferation in low serum medium by monitoring BrdU incorporation. Immunocytochemistry with an anti-BrdU antibody after labeling was used to identify cells that were actively replicating DNA at the time of labeling. Figure 3 shows the percentage of BrdU-positive PC12 cells after 1, 2, and 3 days of treatment under control conditions, with GLP-1 and with NGF. There were no significant differences in proliferation between treated and untreated cells across days. However, PC12 cells showed increased incorporation of BrdU on day 1 after treatment with NGF (9% increase relative to untreated; p < 0.05) and GLP-1 (18% relative to untreated; p < 0.01), and on day 2 after treatment with GLP-1 (10% relative to untreated; p < 0.01). These effects did not translate into an overall sustained effect across days or between treatment groups.

**GLP-1 and Analogs Induce Neurite Outgrowth in PC12 Cells.** PC12 cells, when grown in complete media, develop minimal characteristics of neuronal cell types. When exposed to NGF in low serum medium, the cells stopped dividing and developed morphological properties similar to sympathetic neurons (Fig. 4A). The cells extended long processes, some becoming highly branched, with the cell body exhibiting a more flattened appearance than in cells cultured in low serum medium alone.

Treatment with GLP-1 or exendin-4 in low serum medium produced similar effects on differentiation to those induced by NGF. GLP-1 (Fig. 4B)-mediated neurites, although shorter in length exhibited the same flattened appearance as NGF-mediated neurites. Exendin-4 (Fig. 4C)-mediated neurites were generally much shorter in length than both GLP-1- and NGF-mediated neurites, and were considerably less...
branched, probably as a result of their shorter length. In contrast, the GLP-1 antagonist exendin (9-39) in combination with GLP-1 or exendin-4 failed to initiate neurite extension.

**Treatment with GLP-1 Is Not Associated with Cellular Dysfunction.** We assessed the potentially toxic effects of GLP-1 and analogs in our cell culture system by using two methods: the trypan blue exclusion method and the LDH assay. We did not observe any significant change in viable cell numbers after drug treatment, suggesting that these drugs have no effect on cell proliferation under the conditions studied. To determine the integrity of the cell membrane during treatment, we measured LDH levels quantitatively in the conditioned medium from control and drug-treated cells under the same conditions on day 3. As expected, LDH levels were elevated relative to the media standards (samples were taken at the start of drug treatment) (Fig. 5, A and B). The data presented in Fig. 5 were analyzed separately, because the experiments were conducted at different times. Analysis of variance demonstrated there was no significant main effect of treatment. However, when the analysis was conducted within groups to include individual doses of treatment, the data in Fig. 5A showed an overall significant effect of treatment on levels of LDH ($F_{2,620} = 6.20; p < 0.001$). Tukey’s honestly significant difference test ($Tc = 0.368$ and $0.464$) further revealed two significant effects of treatment on LDH levels compared with controls, $10 \text{ ng/ml NGF}$ resulted in a $1.4$-fold elevation ($p < 0.05$) and $10 \mu \text{g/ml exendin-4}$ resulted in a $1.65$-fold elevation ($p < 0.01$). This is unlikely to be considered “biologically relevant” because any drug treatment within a cell culture system would be expected to induce some degree of disruption of membrane integrity with consequent release of LDH. In support of this, analysis of the data in Fig. 5B revealed there were no significant effects of treatment (between or within groups) on LDH levels compared with controls.

**Inhibition of Neurite Outgrowth with ERK MAP Kinase and PI3-K Inhibitors.** To determine whether GLP-1- and exendin-4-mediated neurite outgrowth was controlled by ERK MAP kinase or PI3-kinase, differentiated cultures were treated with $40 \mu \text{M LY294002}$ (Fig. 4E) or $50 \mu \text{M PD98059}$ (Fig. 4F) to inhibit PI3-kinase and ERK MAP kinase, respectively. Both compounds reduced GLP-1- and exendin-4-mediated neurite outgrowth of the cells. Similarly, NGF-induced neuritic extension was reduced after PD98059 and LY294002 treatment (data not shown). The involvement of both the ERK MAP kinase and the PI3-kinase signaling pathways is thus implicated in GLP-1- and exendin-4-mediated neurite production in PC12 cells.

**Partial Inhibition of Neurite Outgrowth by a PKA Inhibitor.** To determine whether cAMP-dependent MAP kinase phosphorylation was controlled by PKA, GLP-1- and NGF-induced neurites were treated with the PKA-specific inhibitor H89.

Treatment with H89 demonstrated some inhibitory effects on GLP-1- and NGF-mediated neurite outgrowth (data not shown). We infer that PKA is involved in the regulation of the MAP kinase signaling pathway but other signaling pathways must also be involved.

**Quantification of NGF and/or Exendin-4-Mediated Neurite Outgrowth.** To determine the role of exendin-4 and/or NGF on differentiation, daily quantification of neuritic development was carried out. The results shown in Fig. 6 represent the counts taken on day 3 of treatment and are expressed as a percentage of control untreated cells.

Growing PC12 cells in the presence of low serum medium alone resulted in 5 to 10% of neuritic projections. A semiquantitative analysis revealed a significant main effect of drug condition ($F_{4,263.5} = 8.89; p < 0.001$). As expected, NGF treatment significantly induced the neuronal phenotype at the three doses tested herein; $10, 30,$ and $100 \text{ ng/ml}$ NGF (all $p < 0.01$). For example, the treatment of cells with $10$ and $30 \text{ ng/ml NGF}$ produced $550$ and $720\%$ increase in neurite projections from controls, respectively. Under the same conditions when PC12 cells were treated with exendin-4, a sig-

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**Fig. 5.** Fold increases in lactate dehydrogenase levels in the conditioned medium of PC12 cells after treatment with NGF, exendin-4, exendin-4 WOT, and GLP-1. Vertical error bars represent ± standard error of the three separate experimental values. Significant difference from untreated (low serum medium): *, $p < 0.05$; **, $p < 0.01$.

**Fig. 6.** Effect of different concentrations of NGF and/or exendin-4 treatment on neurite outgrowth in PC12 cells. Neurite outgrowth is represented as the percentage of increase in number of cells bearing neurites relative to untreated (low serum medium). Significant difference from untreated: *, $p < 0.05$; **, $p < 0.01$. 

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significant neuritic outgrowth was also observed at 1 μg/ml (98% increase relative to untreated; \( p < 0.05 \)) and 10 μg/ml (160% increase relative to untreated; \( p < 0.01 \)) of the drug. However, the neurite extension with exendin-4 was not as pronounced as that of NGF-treated cells. To determine the synergistic effect of these two drugs, a combination treatment paradigm was tried. When exendin-4 (100 ng/ml) was cotreated with either NGF at 10 or 30 ng/ml, a significant increase in neurite outgrowth was observed relative to untreated control cells (596% increase relative to untreated control cells). Enhancement in neurite outgrowth relative to NGF treatment alone was only significant at 30 ng/ml (\( p < 0.01 \)). Similar positive correlations between dose and the degree of neurite outgrowth were observed with other doses of exendin-4 either alone or in combination with NGF (data not shown). At the doses examined, no ceiling effect was produced, however it is likely that at higher doses of exendin-4 and/or NGF, a maximal effect would be produced and an inverted U-shaped dose-response relationship elicited. Overall, these data suggest that exendin-4 may not initiate differentiation as prolifically as NGF, but exendin-4 can promote NGF-mediated differentiation.

**Exendin-4 Does Not Prevent NGF-Mediated Cell Death.** To investigate possible revival (Fig. 7A) or protective (Fig. 7B) effects of exendin-4 after NGF-mediated cell death, PC12 cells were cultured in the presence of 50 ng/ml NGF (Fig. 7). NGF was withdrawn after 4 or 7 days and the cells were allowed to rejuvenate in complete media. In these experiments NGF withdrawal after 4 days failed to cause massive cell death as has been demonstrated by others, and largely cells were capable of almost fully rejuvenating. Exendin-4 cotreatment did not show significant effects. Withdrawal of NGF after 7 days of treatment caused a 15 to 20% reduction in cell viability, and the cells were not capable of fully rejuvenating (Fig. 7, A and B, column 2). In this case exendin-4 cotreatment did not prevent cell death, at either the low (Fig. 7B, column 4) or the high (Fig. 7B, column 6) dose (1 and 5 μg/ml, respectively).

**Exendin-4 May Rescue Degenerating Cells after NGF-Mediated Withdrawal.** When exendin-4 treatment was carried out after NGF-withdrawal, revival processes were enhanced (Fig. 7A). For example, when PC12 cells were cultured in the presence of NGF for 4 days, NGF was withdrawn and exendin-4 added from days 4 to 7 (Fig. 7A, columns 4 and 6), cell survivability reached untreated control values (>95%). This was the case for both the high (5 μg/ml) and the low (1 μg/ml) dose of exendin-4.

**Effect of GLP-1 and Analogs on Cellular Synaptophysin Levels.** The differences observed in the profile of immunoreactive bands in the immunoblots after drug treatments was due neither to the unequal loading of proteins into the gel nor to the uneven transfer of proteins onto the membrane. Equivalent amounts of total proteins were loaded in each lane of the gel and the efficiency of the electrophoretic transfer was monitored by staining the membranes with Ponceau S.

Western immunoblots of lysates from treated and untreated cells, probed using the synaptophysin antibody, revealed a specific band of approximately 37 kDa (Fig. 8, A and B). Treatment with NGF, GLP-1, and GLP-1 analogs dramatically reduced the expression of the synaptophysin protein compared with control cells. Densitometric quantification of the differences observed in the profile of synaptophysin levels.
the protein bands showed significant reductions for all treatment conditions relative to controls (Fig. 9; all p < 0.01), which appeared to be dose-dependent. No immunoreactive band was detected in conditioned media samples from PC12 cells.

Effect of GLP-1 on Beta-2/NeuroD levels: NeuroD production was determined by Western blot analysis with anti-NeuroD antibody (Fig. 10). A 43-kDa band, apparent in both untreated and GLP-1-treated PC12 cell lysates was detected, which was increased following GLP-1 treatment.

**Discussion**

Our goal in this series of experiments was to study the unique properties of GLP-1 and particular analogs of GLP-1, in a relevant cell culture system. We have presented experimental evidence to demonstrate its role in cell proliferation, neuronal differentiation, and changes in biochemical protein markers, such as synaptophysin and Beta-2/NeuroD. In addition, we have discussed the possible mechanisms of action of GLP-1.

This study has demonstrated that PC12 cells express functional GLP-1 receptors. When activated after treatment with GLP-1, cAMP is rapidly released, reaching maximal levels within 15 min. However, once intracellular stores of cAMP are emptied, levels decrease back to baseline, regardless of the continuous stimulation of the GLP-1 receptor. This classic pulsatile release can be restored after a suitable “recovery phase”, during which time intracellular cAMP stores are replenished.

The dose range of the peptides used in these studies was focused around those currently used in ongoing in vitro experiments relating to pancreatic β-cell function and clinical investigations, and reflects the relative potencies of the peptides for the GLP-1 receptor. Exendin-4 is approximately 100-fold more potent than GLP-1 and the dose range for this peptide is reflected by this higher efficacy. We have compared the effects of GLP-1 and analogs on their ability to induce neurite extension in PC12 cells, and on related signaling mechanisms, by using NGF as a positive control. NGF has been well documented to cause differentiation in PC12 cells through sustained activation of Ras and MAP kinase activity (Qui and Green, 1992; Cowley et al., 1994). In vitro studies with GLP-1 have shown the induction of cell proliferation and differentiation in a broad manner similar to NGF-induced differentiation, not only in PC12 cells but also in the human neuroblastoma cell line SH-SY-5Y (K. T. Y. Shaw, unpublished observations), demonstrating that this phenomenon is not exclusive to PC12 cells. However, the effect of NGF on differentiation was found to be more pronounced than that of GLP-1 and analogs. It is likely that the mechanism of GLP-1 binding was not at the Trk A receptor, as is the case with NGF. The mechanism of GLP-1 and analogs vis-à-vis that of other growth factors such as basic fibroblast growth factor or transforming growth factor remains to be explored.

Neither GLP-1 nor exendin-4 bears sequence homology to NGF and related proteins. The receptors for NGF and neurotrophins (the tyrosine kinase family) and for GLP-1 (a G protein-coupled receptor) have been characterized and are vastly different. It is hence probable that GLP-1 and exendin-4 induce their actions on neuronal, pancreatic, and potentially other cell types via an unrelated mechanism to those of NGF and related proteins. In addition, GLP-1 is structurally smaller than NGF (30 versus 118 amino acids), and thus is far less expensive to synthesize and likely has far greater access to the central and peripheral nervous system after its systemic administration. This is particularly true for longer acting novel analogs of GLP-1 and exendin-4 that have recently been synthesized.

With only a few exceptions, neuropeptides and peptide hormones all act through receptors belonging to the G protein-coupled receptor family. The GLP-1 receptor is present in the brain and pancreas (Campos et al., 1994; Bullock et al., 1996) and it couples positively to the adenylyl cyclase system (Qui and Green, 1992; Cowley et al., 1994). In vitro studies relating to pancreatic cell function and clinical investigations, and reflects the relative potencies of the peptides for the GLP-1 receptor. Exendin-4 is approximately 100-fold more potent than GLP-1 and the dose range for this peptide is reflected by this higher efficacy. We have compared the effects of GLP-1 and analogs on their ability to induce neurite extension in PC12 cells, and on related signaling mechanisms, by using NGF as a positive control. NGF has been well documented to cause differentiation in PC12 cells through sustained activation of Ras and MAP kinase activity (Qui and Green, 1992; Cowley et al., 1994). In vitro studies with GLP-1 have shown the induction of cell proliferation and differentiation in a broad manner similar to NGF-induced differentiation, not only in PC12 cells but also in the human neuroblastoma cell line SH-SY-5Y (K. T. Y. Shaw, unpublished observations), demonstrating that this phenomenon is not exclusive to PC12 cells. However, the effect of NGF on differentiation was found to be more pronounced than that of GLP-1 and analogs. It is likely that the mechanism of GLP-1 binding was not at the Trk A receptor, as is the case with NGF. The mechanism of GLP-1 and analogs vis-à-vis that of other growth factors such as basic fibroblast growth factor or transforming growth factor remains to be explored.

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cAMP-induced differentiation in neuronal cells is generally believed to involve the activation of PKA (Vossler et al.,

**Fig. 9.** Densitometric quantification of synaptophysin proteins extracted from NGF-, exendin-4-, exendin-4 WOT-, and GLP-1-treated PC12 cells. Using Molecular Analyst software the density of the protein bands was compared. The data are presented as the percentage of difference from untreated. Vertical error bars represent ± standard error of three individual experimental values conducted at separate time intervals. Significant difference from untreated; ***, p < 0.01.

**Fig. 10.** Immunodetection of NeuroD in untreated (−) and GLP-1 treated (+) PC12 cells.
1997). Our data have shown that GLP-1 stimulates cAMP formation in PC12 cells. However, because neurite development was only partially inhibited by H89, we suggest that the signaling mechanisms through which GLP-1 and exendin-4 induce differentiation in PC12 cells involves other additional signaling factors. This corroborates recent data from Zhang et al. (1999) who found that endogenous δ-opioid receptors were coupled to the p38 MAP kinase signaling pathway via activation of PKA. However, cAMP may also activate the MAP kinase cascade independently of PKA, through cAMP-regulated guanine nucleotide exchange factors as described by Leech et al. (2000).

Our studies have shown inhibition of GLP-1- and exendin-4-mediated neurite outgrowth after treatment with the PI3-kinase inhibitor LY294002 and the ERK MAP kinase inhibitor PD98059. This suggests a role for both PI3-kinase and ERK MAP kinase pathways in GLP-1- and exendin-4-mediated differentiation in PC12 cells. Support for this is provided by Kita et al. (1998) who have demonstrated neurite extension after microinjection of activated PI3-kinase into PC12 cells. This further corroborates that action of these compounds is through the G protein-coupled GLP-1 receptor, but also implicates the involvement of both the ERK MAP kinase signaling pathway and to a lesser degree the PKA signaling pathway.

Because of the obvious crossover of our compounds from pancreas to brain, we examined the involvement of the transcription factor Beta-2/NeuroD, which plays a major role in both neuronal and pancreatic endocrine development. Expression of NeuroD appears to be transient in sensory and motor neurons of the peripheral nervous system, sensory organs as well as parts of the brain and spinal cord during neuronal differentiation; however, detection in the adult brain may suggest a secondary role in mature neurons (Lee, 1997). Beta-2 expression in pancreatic endocrine cells, the intestine, and the brain activates insulin gene transcription and can induce neurons to differentiate. Mutant mice lacking the functional Beta-2 gene have a striking reduction in the number of insulin-producing β-cells, fail to develop mature islets, and as a consequence develop severe diabetes often resulting in perinatal death (Naya et al., 1997). Thus, Beta-2/NeuroD is essential for in vivo pancreatic development and neuronal differentiation. We have demonstrated that Beta-2/NeuroD expression is increased after treatment with GLP-1, providing further evidence for the neuronal differentiation properties of this insulinotropic peptide. As anticipated, cultures exposed to low serum medium alone showed nominal expression of Beta-2/NeuroD. Indeed, Noma et al. (1999) have shown that overexpression of NeuroD in transfected PC12 cells induced morphological changes such as neurite-like processes and synapse-like structures, without a differentiating-inducing agent such as NFG. Taken together these results suggest that the observed neuronal differentiation induced by GLP-1 and exendin-4 may be mediated by the Beta-2/NeuroD transcription factor.

To examine the molecular changes that occur during differentiation, we have studied the profile of synaptophysin, which is a 37-kDa phosphorylated protein that is highly expressed in the synaptic vesicle membrane. The high degree of differentiation in PC12 cells as a result of NFG treatment was accompanied by a marked decrease in synaptophysin expression relative to untreated control cells. NFG treatment caused a dose-dependent change in cellular synaptophysin expression, producing an approximately 70% maximal decrease relative to control cells. GLP-1 and analogs, which showed similar effects on neurite extension to NFG-mediated differentiation but to a lesser degree, displayed comparatively smaller decreases in synaptophysin expression. Interestingly, NFG and exendin-4 in combination produced a larger decrease in synaptophysin expression than either compound alone, which is consistent with their additive morphological effects (Fig. 9). Overall, exendin-4 showed a more pronounced induction of differentiation in PC12 cells, in terms of synaptophysin expression than did either GLP-1 or exendin-4 WOT. The effect of NFG and the GLP-1 compounds on the expression of synaptophysin is interesting. The anti-synaptophysin antibody allows specific staining of neurosecretory vesicles in pheochromocytoma cells. It may be that during synaptogenesis or neurite formation with NFG or GLP-1 treatment, synthesis of synaptophysin is reduced through yet unknown mechanisms. Indeed, Lahiri and colleagues have shown similar effects on synaptophysin expression after treatment with NFG (Lahiri et al., 2000) and melatonin (Lahiri, 1999), and support the findings in our studies. An alternative explanation may be that the synthesis rate of the protein remains unchanged, but synaptophysin undergoes post-translational modifications, or becomes complexed with other synaptic vesicle protein(s) so that it is inaccessible to antibody detection.

In summary, we have demonstrated that GLP-1 and analogs can induce differentiation in neuronal cells in a manner similar to our prior demonstration in pancreatic cells. These actions are accompanied by biochemical alterations involving the reduction and enhancement of expression of synaptophysin and Beta-2/NeuroD proteins, respectively. Although exendin-4 cotreatment failed to protect against NFG-mediated cell death, our data suggest that exendin-4 may rescue the cell from its degeneration when added alone after NFG withdrawal. These data suggest that GLP-1 and analogs may offer the possibility for rescue of degenerating neurons in either the central or peripheral nervous systems associated with neurodegeneration. Using the novel neurotrophic property of GLP-1 and analogs, we are currently assessing their physiological consequences in an in vivo model.

References
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