0013-7227/03/$15.00/0
Printed in U.S.A.

Endocrinology 144(10):4385–4392

doi: 10.1210/en.2003-0309

Glucagon-Like Peptide-2 Receptor Activation in the Rat Intestinal Mucosa

NATALIE A. WALSH, BERNARDO YUSTA, MARK P. DACAMBRA, YOUNES ANINI, DANIEL J. DRUCKER, AND PATRICIA L. BRUBAKER

Departments of Physiology (N.A.W., M.P.D., Y.A., P.L.B.) and Medicine (D.J.D., P.L.B.), University of Toronto, Toronto, Canada M5S 1A8; and the Banting and Best Diabetes Centre (B.Y., D.J.D.), The Toronto General Hospital, University of Toronto, Toronto, Canada M5G 2C4

Glucagon-like peptide-2 (GLP-2) increases small intestinal growth and function in rodents and human subjects. GLP-2 exerts its effects through a seven-transmembrane domain, G protein-coupled receptor (GLP-2R), stimulating cAMP generation and activating protein kinase A signaling in heterologous cell lines transfected with the GLP-2R. As intestinal cell lines expressing the GLP-2R have not been identified, we developed methods for studying GLP-2R signaling in the rat small intestinal mucosa *in vitro*. Isolated rat intestinal mucosal cells expressed mRNA transcripts for the GLP-2R, as well as for chromogranin A and β-tubulin III, markers for enteroendocrine and neural cells, respectively. cAMP production in response to [Gly^2]GLP-2, a degradation-resistant analog of GLP-2, was maximal at 10^{-11} M (268 ± 93% of control, $P < 0.001$), with reduced cAMP accumulation observed at higher doses. The cAMP response was diminished by pretreatment with 10^{-9} M GLP-2, and was abolished by pretreatment with 10^{-6} M GLP-2 ($P < 0.05$), indicating receptor desensitization. GLP-2 treatment of isolated mucosal cells increased ^{3}H-thymidine incorporation (to 128 ± 8% of controls, $P < 0.05$), and this was prevented by inhibition of the protein kinase A pathway with H89. In contrast, GLP-2 did not affect p44/p42 MAPK phosphorylation or the levels of cytosolic calcium in the mucosal cell preparation. These results provide the first evidence that activation of the endogenous rat mucosal GLP-2 receptor is linked to activation of a cAMP/protein kinase A-dependent, growth-promoting pathway *in vitro*. (*Endocrinology* 144: 4385–4392, 2003)

GLUCAGON-LIKE PEPTIDE-2 (GLP-2) is an intestinal peptide with growth factor-like activity that is secreted by enteroendocrine L cells in response to luminal fat and carbohydrate (1–3). One of the products of prohormone convertase 1-mediated cleavage of proglucagon (4), GLP-2 bears approximately 40% sequence identity to the related proglucagon-derived peptides, glucagon and GLP-1. Treatment of rodents with GLP-2 increases crypt-to-villus height in the small intestine via stimulation of crypt cell proliferation and inhibition of villus cell apoptosis (5, 6). GLP-2 also increases digestive enzyme activity and nutrient absorption, thereby enhancing differentiated intestinal function (7, 8). Furthermore, GLP-2 treatment prevents the villus hypoplasia that results from iv feeding in rats (9) and reduces intestinal damage in murine models of colitis (10, 11) and enteritis (12, 13). Consistent with these findings, GLP-2 administration increased body mass and improved nutrient absorption in a pilot study of human subjects with short bowel syndrome (14). These observations from both human and animal studies have engendered considerable interest in understanding how GLP-2 exerts its effects on the mucosal epithelium *in vivo*.

Studies of the mechanism of action of GLP-2 have been limited by a lack of suitable physiological models for study of GLP-2 receptor (GLP-2R) activation (15). Indeed, an initial report on the intestinotropic effects of GLP-2 indicated that GLP-2 stimulates differentiation but not proliferation in the intestinal epithelial IEC-6 cell line (16), whereas subsequent studies have demonstrated that pharmacological concentrations of GLP-2 induce modest proliferation in baby hamster kidney (BHK) fibroblasts stably transfected with the rat GLP-2R (17). The GLP-2R is a seven-transmembrane spanning G protein-coupled receptor classified as a member of the glucagon receptor family by virtue of its structural similarity to other members of this group, particularly the GLP-1R, with which it shares approximately 50% sequence identity (18, 19). Like the GLP-1R, the transfected GLP-2R has been demonstrated to activate the cAMP pathway. However, studies on GLP-2R signaling have only been conducted in transfected heterologous cell lines (17, 18, 20, 21) as no intestinal cell line has been shown to express the GLP-2R mRNA transcript (15). Furthermore, the identity of the cells expressing the GLP-2R within the intestine is an area under active investigation, as the receptor has been detected in human enteroendocrine cells (15), as well as in murine enteric neurons (22). Thus, the mechanism of GLP-2 action *in vivo* is complex, and likely involves multiple signaling pathways. We now report the development of an *in vitro* model for the study of GLP-2 action and demonstrate that the endogenous rat GLP-2 receptor signals via a cAMP/protein kinase A-dependent pathway in isolated rat intestinal mucosal cells.

Abbreviations: bFGF, Basic fibroblast growth factor; BHK, baby hamster kidney; GAPDH, glyceraldehyde-3-phosphate-dehydrogenase; GLP, glucagon-like peptide; GLP-2R, GLP-2 receptor; HBSS, Hanks' balanced salt solution; IBMX, [isobutylmethyl]xanthine; KIU, Kallikrein-inactivating units.

Materials and Methods

Isolation and characterization of primary rat mucosal cells

The entire small intestine was excised from adult male Fischer 344 rats (175–200 g; Charles River Canada, St. Constant, Québec, Canada). The

intestine was flushed three times with 30 ml of cold Hanks' balanced salt solution (HBSS-low Ca^{2+}/Mg^{2+}) containing freshly added 0.5 mM dithiothreitol. Mucosal cells were collected using a variation of the method described by Flint *et al.* (23). Briefly, the small intestine was everted on a 4-mm plastic rod and sliced on a cold surgical board into 2- to 3-mm fragments. The fragments were placed into 100 ml of cold, oxygenated HBSS for 5 min and then transferred into 150 ml of cold, oxygenated chelating buffer [27 mM Na_3Citrate, 5 mM Na_2HPO_4, 96 mM NaCl, 8 mM KH_2PO_4, 1.5 mM KCl, 0.5 mM dithiothreitol, 20 mM D-sorbitol, 20 mM sucrose, 2 mM glutamine (pH 7.4) at 4 C] and shaken on ice for 20 min to generate cell fraction 1. The residual tissue pieces were then inverted 15 times each in a series of nine 50-ml conical tubes, each containing 20 ml of cold, oxygenated chelating buffer. Fraction 2 was created by combining the cell suspensions from the first three tubes; the suspensions from the next three tubes constituted fraction 3, and fraction 4 consisted of suspensions from the last three tubes. The remaining intestinal fragments were then placed into 50 ml of cold, oxygenated chelating buffer and gently shaken on ice for 10 min to create fraction 5, and the fragments were finally inverted 20 times each in series of nine conical tubes containing 20 ml of cold, oxygenated chelating buffer to generate fractions 6–14. At the end of the cell collection procedure, sections of the residual tissue were fixed in formalin, sectioned, and stained with hematoxylin-eosin for examination. All animal protocols were approved by the University of Toronto Animal Care Committee.

To identify functional properties associated with differentiated epithelial cells, a sucrase assay was performed on isolated intestinal cell fractions. Collected cells (5×10^4) were homogenized in 1 ml of cold 50 mM KH_2PO_4 buffer (pH 7.0). Quantification of sucrase activity (1 U = 1 μmol/min) in the homogenate was based on the chromogenic reaction between glucose, produced upon sucrase hydrolysis of exogenously added sucrose, and a glucose oxidase reagent solution as previously described (7).

To label proliferating cells *in vivo*, male Fischer rats were given an *ip* injection of 200 μCi/kg [^{3}H-methyl]-thymidine (Amersham Life Science, Oakville, Ontario, Canada). Rats were killed after 1 h, and small intestinal mucosal cells were isolated as described above. Fifty thousand live cells (as determined by Trypan Blue exclusion)/ml of chelating buffer from each cell fraction were then added to 3 ml of cold 10% trichloroacetic acid, followed by vigorous vortexing and centrifugation (1300 × *g* for 10 min at 4 C). The supernatant was aspirated and pellets were dissolved by the addition of 0.5 ml of 5% trichloroacetic acid followed by a 15-min incubation at 90 C. Samples were then analyzed for radioactivity (disintegrations per minute).

Total RNA was isolated from intestinal cell fractions (5×10^4 cells/fraction) by the guanidinium thiocyanate method (24), deoxyribonuclease I-treated and reverse transcribed at 42 C for 50 min using random hexamers and SuperScript II (Life Technologies, Inc., Burlington, Ontario, Canada). Aliquots of the first strand reaction (1/10 vol) were used as templates for PCR using *Taq* polymerase (Life Technologies, Inc.). Primer pairs selected for PCR were based on the reported sequences for the rat GLP-2R [sense 5′-TCTCCACTCCCAACAGATGCGTCT-3′, antisense 5′-GATCTCACTCTCTTCCAGAATCTC-3′ (18)], rat proglucagon [sense 5′-TATATACCTCAGGACACGGAGGAGA-3′, antisense 5′-GAAGGATCCATCAGCATGTCT-3′ (25)], rat chromogranin A (sense 5′-GAGGGTCCTCTCCATCCTTC-3′, antisense 5′-CGCCTTCTCCTCTTTCTCCT-3′; GenBank accession no. AF145445), rat neuron-specific class III β-tubulin (sense 5′-GGACCTCAACCACCTTGTGT-3′, antisense 5′-AACATGGCCGTAAACTGCTC-3′; GenBank accession no. AF459021) and rat glyceraldehyde-3-phosphate-dehydrogenase [GAPDH; sense 5′-TCCACCACCCTGTTGCTGTAG-3′, antisense 5′-GACCACAGTCCATGACATCACT-3′ (26)]. Amplification of the GLP-2R cDNA was performed at an annealing temperature of 66 C for 30 cycles, resulting in the generation of a 1543-bp product. Proglucagon, chromogranin A, neuron-specific class III β-tubulin, and GAPDH cDNA amplifications were performed at an annealing temperature of 60 C for 26 cycles, resulting in the generation of 382-, 550-, 498-, and 452-bp products, respectively. GAPDH cDNA amplification was used as an internal control in each RT-PCR experiment. The specificity of each amplification reaction was monitored by control reactions in which reverse transcriptase (but not RNA template) was omitted from the reverse transcriptase reaction mixture, or water alone was added to the reverse transcriptase reaction mix. Analysis of the PCR products was performed by agarose gel electrophoresis followed by visualization by ethidium bromide staining (Chromogranin A, β-tubulin, and GAPDH cDNAs) or Southern blotting (GLP-2R and proglucagon cDNAs), as previously described (15, 27). ^{32}P-labeled cDNA probes did not encompass primer sequences. Final washing conditions for the RT-PCR Southern blots were 15 mM NaCl, 1.5 mM Na citrate, and 0.1% sodium dodecyl sulfate at 65 C.

To determine the levels of IGF-I in the isolated rat mucosal cells, cells from each fraction were homogenized in 1 ml of 1 M acetic acid (pH 3.5) and stored at −70 C before RIA for IGF-I. On the day of the assay, samples were neutralized with 90 μl of 10 N NaOH, centrifuged at 1300 × *g* for 10 min at 4 C. RIA was then performed on the sample supernatant using an IGF-I RIA kit (American Laboratory Products Co., Ltd., Windham, NH) according to the manufacturer's instructions.

Cells from each fraction were also examined for the presence of basic fibroblast growth factor [bFGF (28)]. Cells were collected by centrifugation at 1300 × *g* for 30 min at 4 C, and the pellets were stored at −70 C until assay. On the day of the assay, pellets were homogenized in 0.5 ml of TBS (pH 7.4) containing 0.05% BSA and 0.005% NaN_3 (Sigma, St. Louis, MO). bFGF levels were determined using a bFGF enzyme immunoassay kit (Neogen Corp., Lexington, KY) according to the manufacturer's instructions.

The isolated rat mucosal cells were examined for the presence of GLP-2 by RIA. In brief, cells from each fraction were homogenized in 5 ml of 1 N HCl containing 5% (vol/vol) formic acid, 1% (vol/vol) trifluoroacetic acid and 1% (wt/vol) NaCl. Homogenates were passed through a C18 silica cartridge (C18 Sep-Pak, Waters Associates, Milford MA), and the adsorbed peptides were eluted with 80% (vol/vol) isopropanol/0.1% (vol/vol) trifluoroacetic acid (29). The eluates were collected and stored at −70 C before RIA for GLP-2 using an antiserum (UTTH-7) that recognizes amino acids 25–30 of GLP-2 (29).

Functional responses of isolated rat mucosal cells to GLP-2

Preliminary screening revealed a robust cAMP response to GLP-2 in fraction 12 cells (data not shown). These cells were therefore used in all subsequent assays. Cells (1.5×10^4) were suspended in 1 ml of cold incubation buffer [80 mM NaCl, 100 mM mannitol, 20 mM Tris, 3 mM K_2HPO_4, 1 mM $MgCl_2$, 2 mM glutamine, and 1 mg/ml BSA (pH 7.4)] in borosilicate glass tubes. Cells were then incubated with 100 μM [isobutylmethyl]xanthine (IBMX) containing either vehicle (control), 100 μM forskolin (Sigma), or rat GLP-2, human [Gly^2]GLP-2 (both from NPS-Allelix Pharmaceuticals Inc., Mississauga, Ontario, Canada), or GLP-$1^{7-36NH2}$, exendin-4, glucagon, or human gastric inhibitory polypeptide (all from Bachem California Inc., Torrance, CA), each at 100 nM. Human [Gly^2]-GLP-2 was used in all subsequent studies as it is resistant to degradation by the enzyme dipeptidylpeptidase IV, which is highly expressed in the rat intestinal epithelium (30). Cells were incubated for 30 min at 37 C, and the reaction was stopped by centrifugation at 1300 × *g* for 5 min at 4 C, followed by the addition of 1 ml 100% ethanol at −20 C. Cell fractions were then homogenized, centrifuged (1300 × *g* for 5 min at 4 C) to remove cellular debris, and the supernatant was collected for the determination of cAMP levels using a cAMP RIA Kit (Biomedical Technologies, Stoughton, MA). All cAMP values are expressed relative to control (vehicle), which is defined as 100% and equals 0.61 ± 0.26 pmol cAMP/1.5×10^4 cells across all experiments.

For dose-response and desensitization studies, cells (1.5×10^4) were suspended in 1 ml of DMEM (Life Technologies, Inc., Gaithersburg, MD) containing 100 μM IBMX and the protease inhibitors Trasylol (1 Kallikrein-inactivating units (KIU)/ml; Bayer Inc., Etobicoke, Ontario, Canada) and Diprotin A (100 μM; Calbiochem-Novabiochem, San Diego, CA), and were then incubated with vehicle (control), 100 μM forskolin, or 10^{-12} to 10^{-6} M [Gly^2]-GLP-2 at 37 C with 5% CO_2 for 30 min. In some experiments, cells were incubated (pretreatment) for 60 min in 1 ml of DMEM containing 100 μM IBMX, 1 KIU/ml Trasylol and 100 μM Diprotin A (final concentrations) with or without [Gly^2]-GLP-2 at either 10^{-9} or 10^{-6} M; the reaction was stopped by centrifugation at 1300 × *g* for 5 min at 4 C, the cells were allowed to recover for 10 min in fresh DMEM without [Gly^2]-GLP-2, and the cells then underwent 60-min incubation (treatment) at 37 C in the absence or presence of [Gly^2]-GLP-2 (10^{-9} M) or forskolin (100 μM). For all experiments, cells were extracted and assayed for cAMP as above.

To establish the effects of GLP-2 on ^{3}H-thymidine incorporation *in vitro*, cells were preincubated with vehicle (control) or H89, a protein kinase A inhibitor (10 μM; Sigma) for 10 min at 37 C with 5% CO_2, followed by incubation for 30 min under the same conditions with

vehicle, 10% fetal calf serum (positive control), 10^{-9} M [Gly2]-GLP-2 ± H89, or 100 μM forskolin (Sigma). The reaction was terminated by addition of 3 ml ice-cold 10% trichloroacetic acid, the precipitate was collected and dissolved in 5% trichloacetic acid by boiling for 15 min, and the total dpm was determined by liquid scintillation counting (total dpm was 46,733 ± 1,164 for control cells).

To determine MAPK responses to GLP-2, cells (~3×10^6) were incubated at 37 C for 15 min in 1 ml of DMEM containing 100 mM IBMX, 1 KIU/ml Trasylol, and 100 mM Diprotin A, without (control) or with [Gly2]-GLP-2 at either 10^{-9} or 10^{-6} M, or 10% fetal calf serum (positive control). The reaction was stopped by centrifugation at 1300 × *g* for 20 min at 4 C. The supernatant was discarded, and the cells were homogenized in 1.0 ml of homogenization buffer [25 mM Tris/HCl (pH 7.4), 1 mM sodium orthovanadate, 10 mM sodium pyrophosphate, 10 mM sodium fluoride, 1 mM molybdic acid, 1 mM EGTA, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, and 10 nM okadaic acid]. Homogenates were centrifuged at 13,000 × *g* for 20 min at 4 C. The supernatants were next boiled for 5 min with sample buffer containing β-mercaptoethanol and stored at −70 C until used. Protein concentration was determined using the bicinchoninic acid protein assay (Pierce, Rockford, IL). Forty to 50 μg of cell extract were separated by discontinuous SDS-PAGE and electrotransferred onto Hybond-C membrane (Amersham Pharmacia Biotech, Baie d'Urfé, Quebec, Canada) using standard procedures. The resulting blot was blocked with 5% skim milk in PBS containing 0.1% Tween 20 and next incubated with a 1:1000 dilution in blocking buffer of a polyclonal antibody to p44/42 MAPK phosphorylated at Thr202 and Tyr204 (Cell Signaling Technology, Beverly, MA). Proteins were detected with a secondary antibody conjugated to horseradish peroxidase and an enhanced chemiluminescence commercial kit (Amersham Pharmacia Biotech). To monitor loading and transfer conditions blots were reprobed with an antiactin polyclonal antibody (Sigma, 1:5000 dilution).

Calcium responses to GLP-2 were examined by suspending 10^5 cells in phosphate-free HBSS containing 2.5 mM $CaCl_2$ and incubation with 2.5 μM fura-2 AM ester (Molecular Probes, Eugene, OR) at 37 C for 30 min. Cells were then washed twice with buffer and incubated at 37 C for an additional 30 min, and fura-2 fluorescence ratio measurements were made on a Hitachi F-2000 fluorescence spectrometer, (excitation: 346 nm; emission: 510 nm). Calibration was performed at 37 C with ionophore A23187 (Sigma) and $MnCl_2$ using the Grynkiewicz equation (31) with a dissociation constant of 224 nM. [Gly^2]-GLP-2 was added to the cell suspension to final concentrations of 10^{-11}, 10^{-9}, and 10^{-7} M.

Data analysis

Data are expressed as mean ± SEM. Statistical differences between groups were assessed by ANOVA on SAS software (Statistical Analysis System, Cary, NC), using n-1 *post hoc* custom hypotheses tests.

Results

To investigate the characteristics of GLP-2R activation in the rat intestine, we developed a method for collection of primary rat small intestinal cells, using a calcium chelator in combination with mechanical dispersion to generate a series of 14 cell fractions. Morphometric analysis of the tissue fragments remaining after cell dispersal demonstrated that this procedure mainly results in the liberation of villus and crypt cells with minimal effects on cells in the underlying muscularis. A few fragments of residual mucosal tissue were identified in some experiments after the fractionation process (Fig. 1A). To functionally characterize the mucosal cell preparation, cells from fractions 1–14 were assayed for sucrase activity, an enzymatic activity that is characteristic of differentiated enterocytes (32). Sucrase levels were highest in fraction 2 ($P < 0.01$ *vs.* fraction 14) and decreased toward the later fractions (Fig. 1B). To functionally identity crypt cells, which are located in the proliferative compartment of the small intestine (32), rats were injected *in vivo* with ^{3}H-thymidine 1 h before isolation of the cells. Analysis of the ^{3}H-DNA content of these cells demonstrated peak levels in fraction 13, which were 650-fold greater than those in fraction 2 ($P < 0.05$). These results indicate that the isolated rat mucosal cell fractions contain a functional gradient of cell types.

To study the effects of GLP-2 *in vitro*, we first determined whether GLP-2R mRNA transcripts were present in isolated rat mucosal cells by RT-PCR analysis of RNA from fractions 4, 7, 12, and 13. GLP-2R mRNA transcripts were detected in all fractions tested, as were transcripts for markers of both enteroendocrine cells (chromogranin A) and enteric neurons (β-tubulin III; Fig. 1C). Consistent with the presence of enteroendocrine cells in the cell preparation, proglucagon mRNA transcripts were also detected in each of these fractions. Furthermore, GLP-2 RIA carried out on mucosal cell extracts revealed the presence of immunoreactive GLP-2 in all fractions (Fig. 1D). Similarly, lower but detectable levels of several other known intestinal growth factors, IGF-I and bFGF (33), were also detected in mucosal cells extracts (Fig. 1D). Although levels of immunoreactive IGF-I were fairly evenly distributed across the cell fractions, bFGF was only detected in fractions 7–14.

To determine whether isolated rat mucosal cells exhibit a functional response to GLP-2, cells were incubated with GLP-2, and the production of cAMP was determined (Fig. 2). Treatment of the isolated cells with either GLP-2 or its dipeptidylpeptidase IV-resistant analog, [Gly^2]-GLP-2, stimulated a significant increase in cAMP levels (10^{-7} M each; to 135 ± 8% and 140 ± 11% of control, respectively, $P < 0.01$–0.001). The specificity of this response was tested in the same experiments, using several structurally related members of the glucagon peptide superfamily. No changes in cAMP levels were detected in the cells following incubation with GLP-1, exendin-4 (a GLP-1 homolog), glucagon, or gastric inhibitory polypeptide (each at 10^{-7} M; Fig. 2).

In dose-response studies, GLP-2-stimulated cAMP production by the mucosal cells was maximal at 10^{-11} M [Gly^2]-GLP-2 (268 ± 93% of controls, $P < 0.001$; Fig. 3A). Conversely, higher concentrations of GLP-2, ranging from 10^{-9} to 10^{-7} M, produced a maximum response of 163 ± 31% of controls (10^{-9} M, $P < 0.05$), whereas treatment with 10^{-6} M GLP-2 did not result in increased cAMP accumulation. These findings are consistent with previous dose-response relationships observed with the related proglucagon-derived peptide, GLP-1, in insulinoma cells (34). To determine whether prior exposure to GLP-2 diminished the subsequent cAMP response to rechallenge, isolated mucosal cells were pretreated with either vehicle alone or with [Gly^2]-GLP-2 at a concentration of either 10^{-9} or 10^{-6} M for 60 min, followed by a brief recovery and then stimulation with vehicle alone, 100 μM forskolin (positive control) or 10^{-9} M [Gly^2]-GLP-2. Cells pretreated with vehicle responded to GLP-2 with cAMP accumulation comparable to that attained in the dose response studies. However, the cAMP response of cells pretreated with 10^{-9} M [Gly^2]-GLP-2 was diminished compared with that of cells pretreated with vehicle (Fig. 3B), whereas the response of cells to treatment with 10^{-9} M [Gly^2]-GLP-2 was completely abrogated by pretreatment with 10^{-6} M [Gly^2]-GLP-2 ($P < 0.05$, Fig. 3C). When taken together, these results indicate that pretreatment with GLP-2 attenuates the

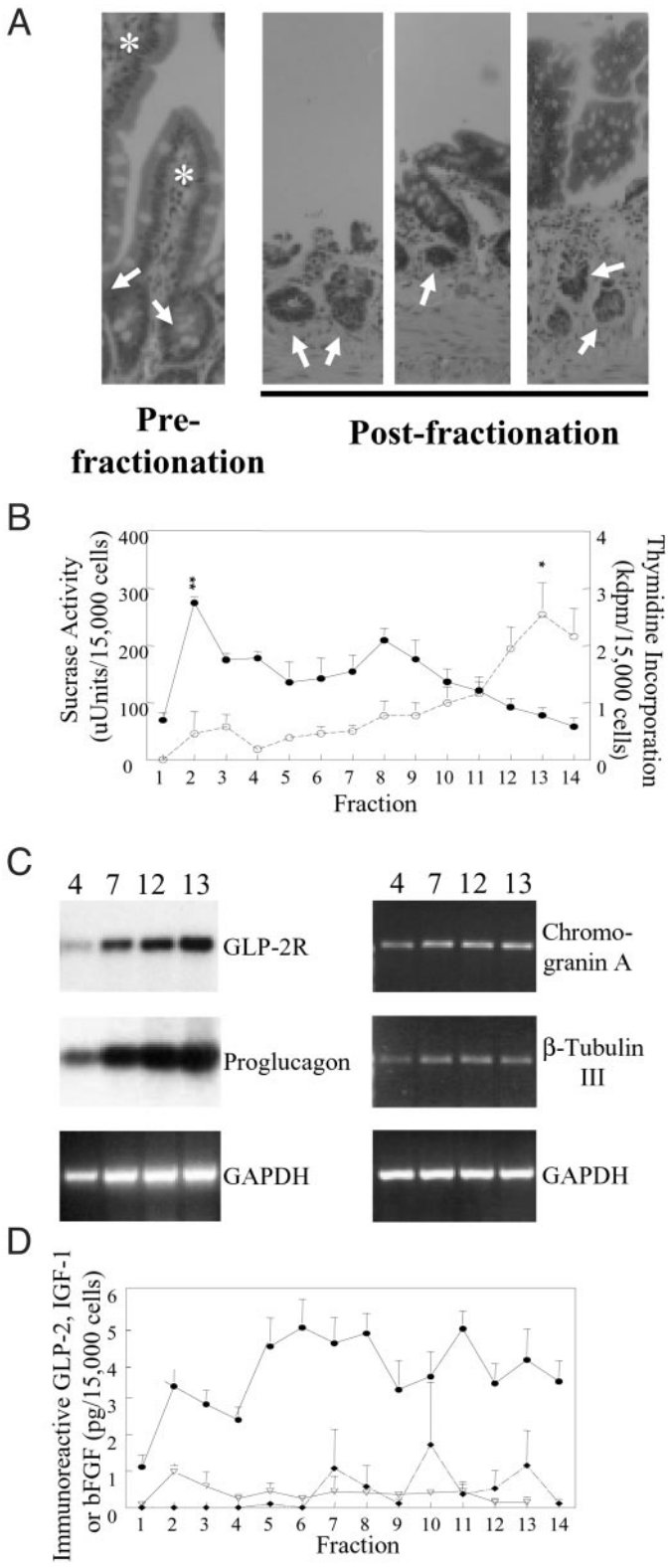

FIG. 1. Characterization of isolated rat small intestinal mucosal cells. A, Photomicrographs (×200) of control rat small intestine (*left panel*) and the residual tissue after collection of the mucosal cell fractions (*right panels*). The *asterisks* indicate villi, and the *arrows* indicate crypts. B, Sucrase activity (*solid line*), and ^{3}H-thymidine levels (*dotted line*) in isolated rat small intestinal mucosal cells. Fourteen cell fractions were isolated and assayed for either sucrase activity (determined as micrograms glucose liberated from sucrose; n = 4) or [^{3}H-methyl]thymidine incorporation 1 h post *in vivo* injection (n = 3). *, $P < 0.05$ for fraction 1 *vs.* 13; **, $P < 0.01$ for fraction 2 *vs.* 14 (kdpm: thousands of disintegrations per minute). C, RT-PCR of GLP-2R, proglucagon, chromogranin A, β-tubulin III, and GAPDH (control) mRNA transcripts in total cellular RNA isolated from rat small intestinal mucosal cells (fractions 4, 7, 12, and 13). D, GLP-2, IGF-I, and bFGF content of isolated rat small intestinal mucosal cells. Fractions 1–14 were assayed for: GLP-2 by RIA (●; n = 6); IGF-I using an RIA kit (▽; n = 3); and bFGF using an EIA kit (◆; n = 4).

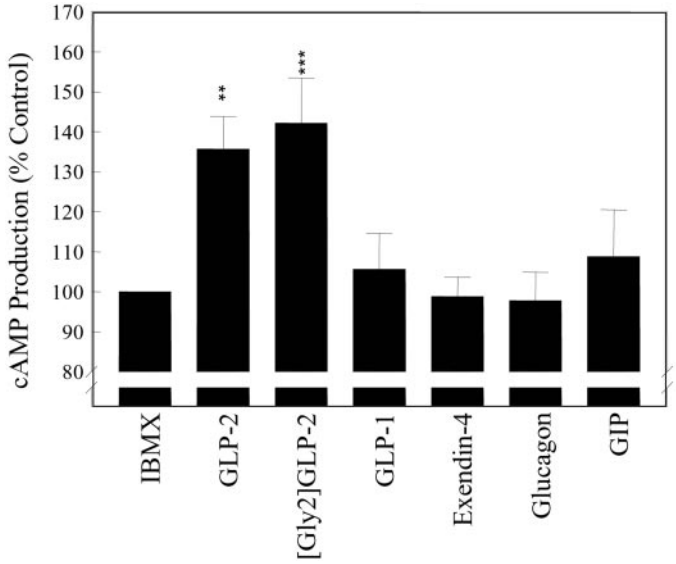

FIG. 2. Specificity of the isolated rat small intestinal mucosal cell (fraction 12) response to GLP-2. All media contained 100 μM IBMX. Cells (1.5×10^4) were incubated for 30 min with media alone or media + 10^{-7} M peptide, and cAMP production was determined by RIA (n = 6). **, $P < 0.01$; ***, $P < 0.001$ *vs.* control.

functional response of the isolated rat intestinal mucosal cells to supraphysiologic doses of GLP-2.

GLP-2 is known to exert tropic effects on the gastrointestinal epithelium *in vivo* (5, 6, 9–13). Therefore, to examine one component of a cellular proliferative response, the isolated mucosal cells were incubated with ^{3}H-thymidine for 30 min (Fig. 4A). Addition of 10% fetal calf serum increased ^{3}H-thymidine incorporation to 162 ± 8% of control values ($P < 0.001$), whereas [Gly^2]-GLP-2 (10^{-9} M) increased incorporation to 128 ± 8% of control ($P < 0.01$). Preincubation of the cells with the protein kinase A inhibitor, H89, completely abrogated the stimulatory effect of GLP-2 on ^{3}H-thymidine incorporation (107 ± 6% of control; $P < 0.05$ *vs.* [Gly^2]-GLP-2 alone). In contrast, incubation of the cells with forskolin had no effect on ^{3}H-thymidine incorporation.

To determine whether the MAPK signaling pathway was activated by GLP-2 in the isolated cells, the levels of phosphorylated p44 and p42 MAPK were determined by Western blot (Fig. 4, B and C). [Gly^2]-GLP-2 (10^{-9} M) failed to activate p44/p42 MAPK when applied directly to the cells. In contrast, MAPK was phosphorylated in response to incubation with 10% fetal calf serum (Fig. 4, B and C). Thus, although GLP-2 treatment increased ^{3}H-thymidine incorporation in a protein kinase A-dependent fashion, this was not associated with activation of p44/p42 MAPK.

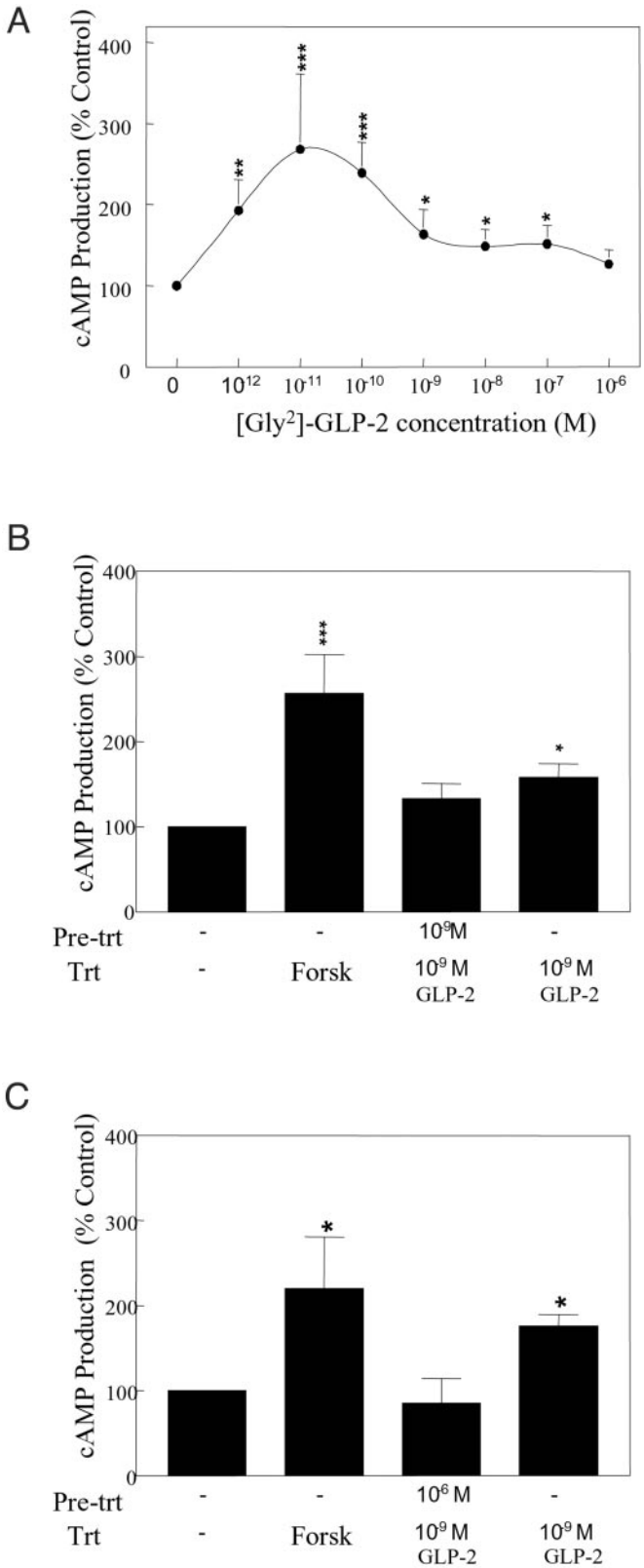

FIG. 3. Dose response and desensitization of isolated rat small intestinal mucosal cells (fraction 12) to GLP-2. All media contained 100 μM IBMX. A, Cells (1.5×10^4) were incubated for 30 min with media alone or media + 10^{-12}–10^{-6} M human [Gly^2]-GLP-2, and cAMP production was determined by RIA (n = 9). B, Cells (1.5×10^4) were pretreated (Pre-trt) for 60 min with media alone (−) or media + 10^{-9} M human [Gly2]-GLP-2. Cells were collected by centrifugation at $1300 \times g$, allowed to recover for 10 min in fresh medium, and then treated (Trt) for 60 min with media alone, media +100 μM forskolin (Forsk, positive control), or media + 10^{-9} M human [Gly^2]-GLP-2 (n = 4 each). C, Experiments were carried out as in (B), but with 10^{-6} M human [Gly^2]-GLP-2 in the Pre-trt (n = 4 each). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ *vs.* control.

As previous studies have shown that that the highly related GLP-1 and glucagon receptors exhibit dual signaling through both cAMP- and calcium-dependent pathways (35–37), we assessed whether GLP-2R signaling in the mucosal cells was coupled to changes in $[Ca^{2+}]_c$. Mucosal cells were preloaded with fura-2 AM ester and subjected to spectrofluorometric analysis in the presence or absence of GLP-2. [Gly^2]-GLP-2 did not increase $[Ca^{2+}]_c$ in rat mucosal cells at any dose tested (Fig. 5). However, the cells were responsive to the nonspecific calcium ionophore, A23187 (12.5 μM), and this response was appropriately quenched by addition of $MnCl_2$ (6 mM). The responses to A23187 and $MnCl_2$ were used to calculate the resting $[Ca^{2+}]_c$ of the rat mucosal cells as 50 ± 20 nM.

Discussion

In the current study we report, for the first time, that the endogenous rat GLP-2R is expressed in isolated intestinal mucosal cells. Consistent with data obtained from studies of GLP-2 receptor signaling in transfected heterologous cells (17, 18, 20, 21), ligand-induced activation of the endogenous rat GLP-2R was found to be linked to production of cAMP. Furthermore, no cAMP accumulation was detected in response to incubation with related members of the glucagon peptide superfamily. These findings suggest a high degree of specificity of the GLP-2R for its ligand, both in primary intestinal cells and when expressed into heterologous fibroblasts (17, 18, 38). However, although the GLP-2R displayed a dose-response relationship to GLP-2 in both types of cells, the sensitivity of the endogenous intestinal mucosal GLP-2R to activation differed markedly from that reported for the transfected receptor. In COS cells transfected with the rat GLP-2R, maximum activation is attained by GLP-2 concentrations ranging from 10^{-9} to 10^{-8} M, whereas lower concentrations fail to produce a significant response (18). Similarly, the cloned rat GLP-2R expressed in BHK cells demonstrates a low response to 10^{-11} to 10^{-10} M [Gly^2]-GLP-2, and is maximally activated by 10^{-9} to 10^{-7} M [Gly^2]-GLP-2 (17, 21, 38). By contrast, the endogenous rat GLP-2R studied here was maximally activated by [Gly^2]-GLP-2 at 10^{-11} M and responded less strongly to all doses of ligand between 10^{-12} and 10^{-7} M.

Similar differences have also been noted for activation of the GLP-1R by its ligand in cells expressing the endogenous receptor compared with heterologous cells transfected with the receptor [*e.g.* ED_{50}s of 0.2 and 2 nM, respectively (20, 21)]. Such discrepancies in the dose-response profiles of the endogenous and transfected receptors may result from different numbers of receptors expressed by each cell. Correlations between receptor number and dose-dependent activation have been demonstrated using transfected Chinese hamster

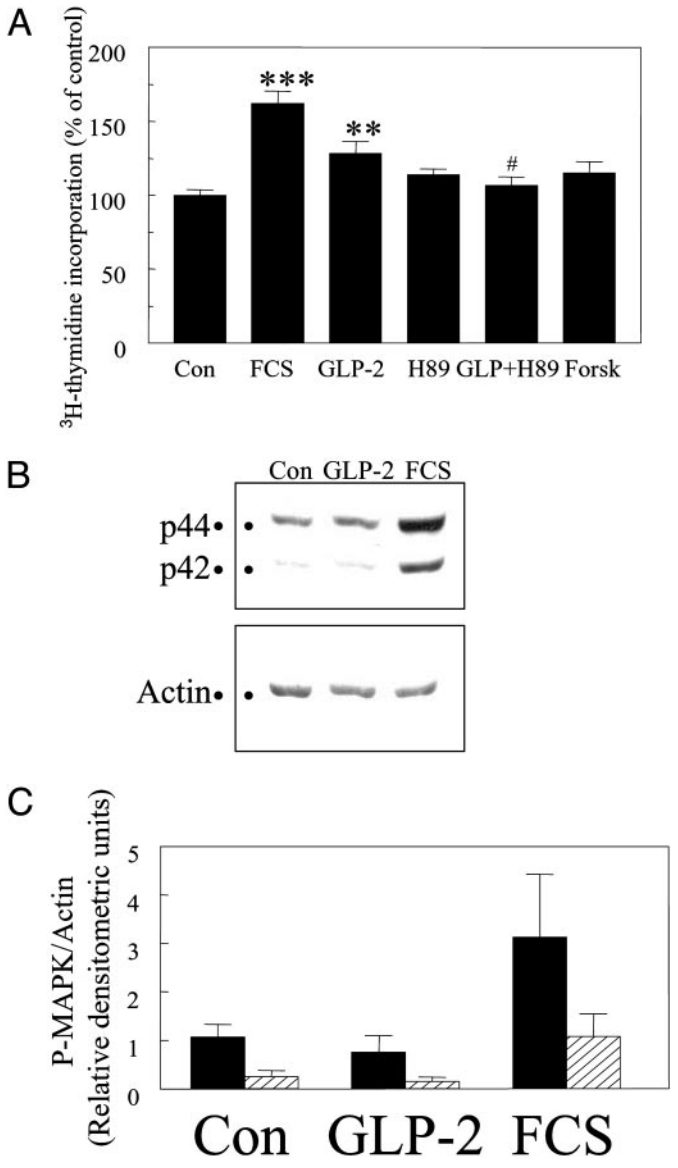

FIG. 4. Effects of GLP-2 on ^{3}H-thymidine incorporation and MAPK activation in rat small intestinal mucosal cells. A, Cells were preincubated with vehicle (Con) or 10 μM H89 for 10 min, followed by incubation with ^{3}H-thymidine in vehicle, 10% fetal calf serum (FCS), 10^{-9} M human [Gly^2]-GLP-2 ± H89 or 100 μM forskolin (Forsk) for 30 min (n = 8). **, $P < 0.01$; ***, $P < 0.001$ *vs.* control; #, $P < 0.05$ *vs.* GLP-2 alone. B, Representative Western blot showing phosphorylated p44 and p42 MAPK and actin levels in rat mucosal cells treated with media alone (Con), or media containing either 10^{-9} M [Gly^2]-GLP-2 or 10% FCS. C, Western blots were quantitated by densitometry, and the results are shown for total phosphorylated p44 (*solid bars*) and p42 (*hatched bars*) MAPK relative to actin levels (n = 3).

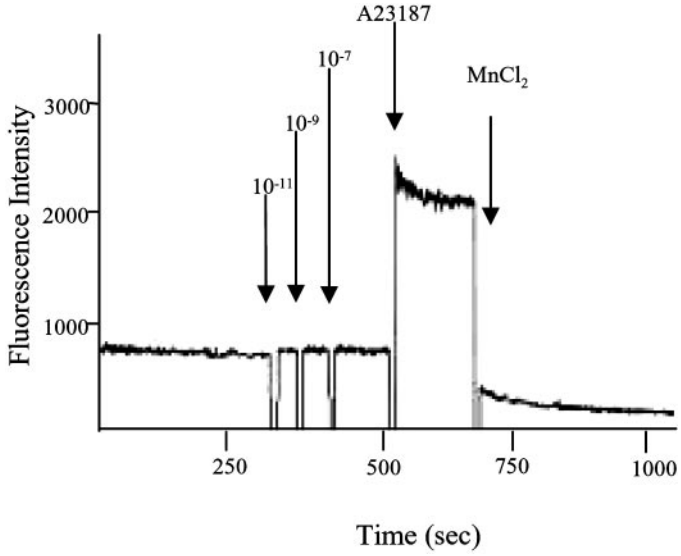

FIG. 5. Cytosolic calcium in isolated rat small intestinal mucosal cells. Cells (10^5) were suspended in phosphate-free HBSS containing 2.5 mM $CaCl_2$ and loaded with 2.5 μM fura-2 AM ester. Fura-2 fluorescence ratio measurements were made with excitation at 346 nm and emission at 510 nm before and after the addition of human [Gly^2]-GLP-2 to final concentrations of 10^{-11}, 10^{-9}, and 10^{-7} M. Calcium ionophore A23187 (12.5 μM) was used both as a positive control and for calibration. $MnCl_2$ (6 mM) was also used in the calibration process. The trace shown is representative of three independent experiments. Fluorescence values are expressed in arbitrary fluorescence units.

ovary cells expressing different levels of the GLP-1R. Cells expressing lower receptor numbers produced less cAMP but required much lower doses of GLP-1 to attain maximum activation (39). Thus, it appears that low receptor expression by cells can confer a high degree of ligand sensitivity. Furthermore, it is likely that not all of the isolated primary mucosal cells express the GLP-2R, whereas transfected cell lines are selected such that all cells express the receptor. These findings therefore suggest that at least one explanation for the difference between the results obtained in the present study with the endogenous rat GLP-2R and data obtained in studies of the transfected GLP-2R may relate to differing expression levels of the receptor protein.

It is interesting to note that the dose of [Gly^2]-GLP-2 that maximally activated the endogenous rat GLP-2R is close to the circulating concentration of GLP-2 found in plasma from fasted rats [5×10^{-11} M (29, 40)] and humans [10^{-12} M (1, 3)]. Given that the plasma concentration of GLP-2 (1–33) in humans increases 3- to 6-fold within 60 min of ingestion of carbohydrate or fat (1, 3), our results suggest that the mucosal rat GLP-2R is capable of activation by the full spectrum of physiologic GLP-2 concentrations.

The GLP-2R appears to undergo homologous desensitization as pretreatment of cells with 10^{-6} M [Gly^2]-GLP-2 completely abrogated cAMP production in response to subsequent stimulation with [Gly^2]-GLP-2. Agonist-induced desensitization of G protein-coupled receptors has been shown for several receptors that share a high degree of homology with the GLP-2R, including those for GLP-1 (34, 41–43) and glucagon (43, 44). GLP-1R studies in the β-cell line HIT-T15 have demonstrated that exposure to 10^{-7} M GLP-1 followed by a short recovery period significantly reduces both cAMP accumulation and insulin secretion upon restimulation with 10^{-8} M GLP-1 (34). Biochemical studies of the mechanisms mediating these effects have demonstrated that homologous desensitization of the rat GLP-1R requires phosphorylation at three serine doublets in the C-terminal tail of the receptor (43). The presence of similar serine doublets in the C-terminal tail of the rat GLP-2R (18) raises the possibility that the homologous desensitization reported here may occur by a similar mechanism. Alternatively, it has been reported that

the related PTH receptor can couple to multiple G proteins, each with a distinct dose-response curve for activation of downstream signaling pathways (45). Furthermore, very recent studies have demonstrated that treatment of neurons expressing the GnRH receptor with high concentrations of ligand induces a switch in G protein coupling, changing the signal from Gs-cAMP stimulatory to Gi-cAMP inhibitory (46). Further studies to elucidate the mechanism(s) underlying GLP-2R desensitization are currently underway.

The intestinotropic effects of GLP-2 *in vivo* are exerted, in part, through stimulation of crypt cell proliferation (5, 6). In the present study, GLP-2 treatment was found to induce a modest, but significant increase in incorporation of ^{3}H-thymidine into the isolated cells. Whether GLP-2 can actually increase cell numbers in this model was not examined, as long-term maintenance of cells isolated from the adult rat intestine is rendered difficult due to bacterial contamination. Importantly, however, these studies demonstrate for the first time that GLP-2-induced thymidine incorporation into intestinal mucosal cells was prevented by inhibition of protein kinase A. This finding is consistent with the ability of GLP-2 to enhance cAMP levels in these cells, but stands in contrast to the finding that forskolin treatment did not stimulate thymidine incorporation in the same cells. Nonetheless, these results are remarkably similar to findings made in the BHK-GLP-2R cell line, in which treatment with GLP-2, but not with 8-Br-cAMP, increased cell numbers (17). Furthermore, GLP-2 failed to increase MAPK phosphorylation in both isolated rat mucosal cells and BHK-GLP-2R cells (17), suggesting that the proliferative responses to GLP-2 in these *in vitro* models are not linked to activation of this growth-related kinase pathway. Whether GLP-2 directly stimulates cell proliferation or acts in part through enhancement of the activity of other growth factors present in the isolated cells, such as IGF-I and/or bFGF, is an important factor for future consideration.

GLP-2 did not alter calcium levels in the isolated rat mucosal cells, despite the ability of these cells to respond to a nonspecific calcium ionophore. The induction of calcium signaling by the related GLP-1 (35, 36) and glucagon (37) receptors, combined with the structural homology of these receptors to the GLP-2R (18), was the rationale for testing the ability of the GLP-2R to signal through calcium. However, the lack of calcium signaling by GLP-2 in the intestinal cells is consistent with the finding that the transfected GLP-2R also fails to activate calcium signaling in fibroblasts (17).

In summary, we report that [Gly^2]-GLP-2 activates cAMP production in a specific dose-dependent manner in isolated rat small intestinal mucosal cells that express the GLP-2R. Physiologic doses of the peptide stimulate cAMP production and enhance ^{3}H-thymidine uptake in a protein kinase A-dependent fashion; however, the endogenous rat GLP-2R does not couple to calcium or, importantly, to p44/p42 MAPK in the isolated cells. These findings provide a new model for future studies on the identification of the downstream mediators of intestinal GLP-2 activity in the non-transformed rodent epithelium.

Acknowledgments

The authors are grateful to NPS-Allelix Inc. (Mississauga, Ontario, Canada) for the gift of human [Gly2]-GLP-2, to Dr. A. Klip (University of Toronto) for assistance with the MAPK studies, and to Dr. G. Downey (University of Toronto) for assistance with the calcium studies.

Received March 10, 2003. Accepted June 24, 2003.

Address all correspondence and requests for reprints to: Dr. P. L. Brubaker, Room 3366, Medical Sciences Building, University of Toronto, Toronto, Ontario, Canada M5S 1A8. E-mail: p.brubaker@utoronto.ca.

This work was supported by grants from the Canadian Institutes of Health Research (CIHR) (to P.L.B. and D.J.D.) and the National Cancer Institute of Canada (D.J.D.). M.D. was supported by a Graduate Scholarship from the Department of Physiology, University of Toronto, and Y.A. was supported by a postdoctoral fellowship from the Canadian Diabetes Association. D.J.D. is a Senior Scientist of the CIHR and a consultant to NPS-Allelix, Inc., and P.L.B. is supported by the Canada Research Chairs Program.

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