

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

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**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  $\beta$ -tubulin III, markers for enteroendocrine and neural cells, respectively. cAMP production in response to [Gly<sup>2</sup>]GLP-2, a degradation-resistant analog of GLP-2, was maximal at  $10^{-11}$  M ( $268 \pm 93\%$  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 <sup>3</sup>H-thymidine incorporation (to  $128 \pm 8\%$  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)**

**G**LUCAGON-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.

## 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

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.

intestine was flushed three times with 30 ml of cold Hanks' balanced salt solution (HBSS-low  $\text{Ca}^{2+}$ / $\text{Mg}^{2+}$ ) 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  $\text{Na}_3\text{Citrate}$ , 5 mM  $\text{Na}_2\text{HPO}_4$ , 96 mM  $\text{NaCl}$ , 8 mM  $\text{KH}_2\text{PO}_4$ , 1.5 mM  $\text{KCl}$ , 0.5 mM dithiothreitol, 20 mM  $\text{D-sorbitol}$ , 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 sucrose assay was performed on isolated intestinal cell fractions. Collected cells ( $5 \times 10^4$ ) were homogenized in 1 ml of cold 50 mM  $\text{KH}_2\text{PO}_4$  buffer (pH 7.0). Quantification of sucrose activity (1 U = 1  $\mu\text{mol}/\text{min}$ ) in the homogenate was based on the chromogenic reaction between glucose, produced upon sucrose 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  $\mu\text{Ci}/\text{kg}$  [ $^3\text{H}$ -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 ( $1300 \times g$  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 ( $5 \times 10^4$  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'-GATCTACTCTCTCCAGAAATCTC-3' (18)], rat proglucagon [sense 5'-TATATACCTCAGGACAGGAGGAGA-3', antisense 5'-GAAGGATCCATCAGCATGTCT-3' (25)], rat chromogranin A (sense 5'-GAGGGTCTCTCCATCCTTC-3', antisense 5'-CGCCTTCTCTTCTCTCTCT-3'; GenBank accession no. AF145445), rat neuron-specific class III  $\beta$ -tubulin (sense 5'-GGACCTCAACCACCTTGTTGT-3', antisense 5'-AATCATGGCCGTAAACTGCTC-3'; GenBank accession no. AF459021) and rat glyceraldehyde-3-phosphate-dehydrogenase [GAPDH; sense 5'-TCCACACCCTGTGCTGTAG-3', antisense 5'-GACCACAGTCCATGACATCCT-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  $\beta$ -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,  $\beta$ -tubulin, and GAPDH cDNAs) or Southern

blotting (GLP-2R and proglucagon cDNAs), as previously described (15, 27).  $^{32}\text{P}$ -labeled cDNA probes did not encompass primer sequences. Final washing conditions for the RT-PCR Southern blots were 15 mM  $\text{NaCl}$ , 1.5 mM  $\text{Na citrate}$ , 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  $\mu\text{l}$  of 10 N  $\text{NaOH}$ , centrifuged at  $1300 \times g$  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  $1300 \times g$  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%  $\text{NaN}_3$  (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  $\text{HCl}$  containing 5% (vol/vol) formic acid, 1% (vol/vol) trifluoroacetic acid and 1% (wt/vol)  $\text{NaCl}$ . 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 ( $1.5 \times 10^4$ ) were suspended in 1 ml of cold incubation buffer [80 mM  $\text{NaCl}$ , 100 mM mannitol, 20 mM Tris, 3 mM  $\text{K}_2\text{HPO}_4$ , 1 mM  $\text{MgCl}_2$ , 2 mM glutamine, and 1 mg/ml BSA (pH 7.4)] in borosilicate glass tubes. Cells were then incubated with 100  $\mu\text{M}$  [isobutylmethyl]xanthine (IBMX) containing either vehicle (control), 100  $\mu\text{M}$  forskolin (Sigma), or rat GLP-2, human [Gly<sup>2</sup>]GLP-2 (both from NPS-Allelix Pharmaceuticals Inc., Mississauga, Ontario, Canada), or GLP-1<sup>7–36</sup>NH<sub>2</sub>, exendin-4, glucagon, or human gastric inhibitory polypeptide (all from Bachem California Inc., Torrance, CA), each at 100 nM. Human [Gly<sup>2</sup>]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  $1300 \times g$  for 5 min at 4 C, followed by the addition of 1 ml 100% ethanol at  $-20$  C. Cell fractions were then homogenized, centrifuged ( $1300 \times g$  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  $0.61 \pm 0.26$  pmol cAMP/ $1.5 \times 10^4$  cells across all experiments.

For dose-response and desensitization studies, cells ( $1.5 \times 10^4$ ) were suspended in 1 ml of DMEM (Life Technologies, Inc., Gaithersburg, MD) containing 100  $\mu\text{M}$  IBMX and the protease inhibitors Trasylol (1 Kallikrein-inactivating units (KIU)/ml; Bayer Inc., Etobicoke, Ontario, Canada) and Diprotin A (100  $\mu\text{M}$ ; Calbiochem-Novabiochem, San Diego, CA), and were then incubated with vehicle (control), 100  $\mu\text{M}$  forskolin, or  $10^{-12}$  to  $10^{-6}$  M [Gly<sup>2</sup>]GLP-2 at 37 C with 5%  $\text{CO}_2$  for 30 min. In some experiments, cells were incubated (pretreatment) for 60 min in 1 ml of DMEM containing 100  $\mu\text{M}$  IBMX, 1 KIU/ml Trasylol and 100  $\mu\text{M}$  Diprotin A (final concentrations) with or without [Gly<sup>2</sup>]GLP-2 at either  $10^{-9}$  or  $10^{-6}$  M; the reaction was stopped by centrifugation at  $1300 \times g$  for 5 min at 4 C, the cells were allowed to recover for 10 min in fresh DMEM without [Gly<sup>2</sup>]GLP-2, and the cells then underwent 60-min incubation (treatment) at 37 C in the absence or presence of [Gly<sup>2</sup>]GLP-2 ( $10^{-9}$  M) or forskolin (100  $\mu\text{M}$ ). For all experiments, cells were extracted and assayed for cAMP as above.

To establish the effects of GLP-2 on  $^3\text{H}$ -thymidine incorporation *in vitro*, cells were preincubated with vehicle (control) or H89, a protein kinase A inhibitor (10  $\mu\text{M}$ ; Sigma) for 10 min at 37 C with 5%  $\text{CO}_2$ , followed by incubation for 30 min under the same conditions with

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

To determine MAPK responses to GLP-2, cells ( $\sim 3 \times 10^6$ ) were incubated at 37 C for 15 min in 1 ml of DMEM containing 100 mM IBMX, 1 KIU/ml Trasyolol, and 100 mM Diprotin A, without (control) or with [Gly<sup>2</sup>]-GLP-2 at either  $10^{-9}$  or  $10^{-6}$  M, or 10% fetal calf serum (positive control). The reaction was stopped by centrifugation at  $1300 \times g$  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 molybdc acid, 1 mM EGTA, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, and 10 nM okadaic acid]. Homogenates were centrifuged at  $13,000 \times g$  for 20 min at 4 C. The supernatants were next boiled for 5 min with sample buffer containing  $\beta$ -mercaptoethanol and stored at  $-70$  C until used. Protein concentration was determined using the bicinchoninic acid protein assay (Pierce, Rockford, IL). Forty to 50  $\mu$ g 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 reprobated 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<sub>2</sub> and incubation with 2.5  $\mu$ M 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<sub>2</sub> using the Grynkiewicz equation (31) with a dissociation constant of 224 nM. [Gly<sup>2</sup>]-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  $\pm$  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

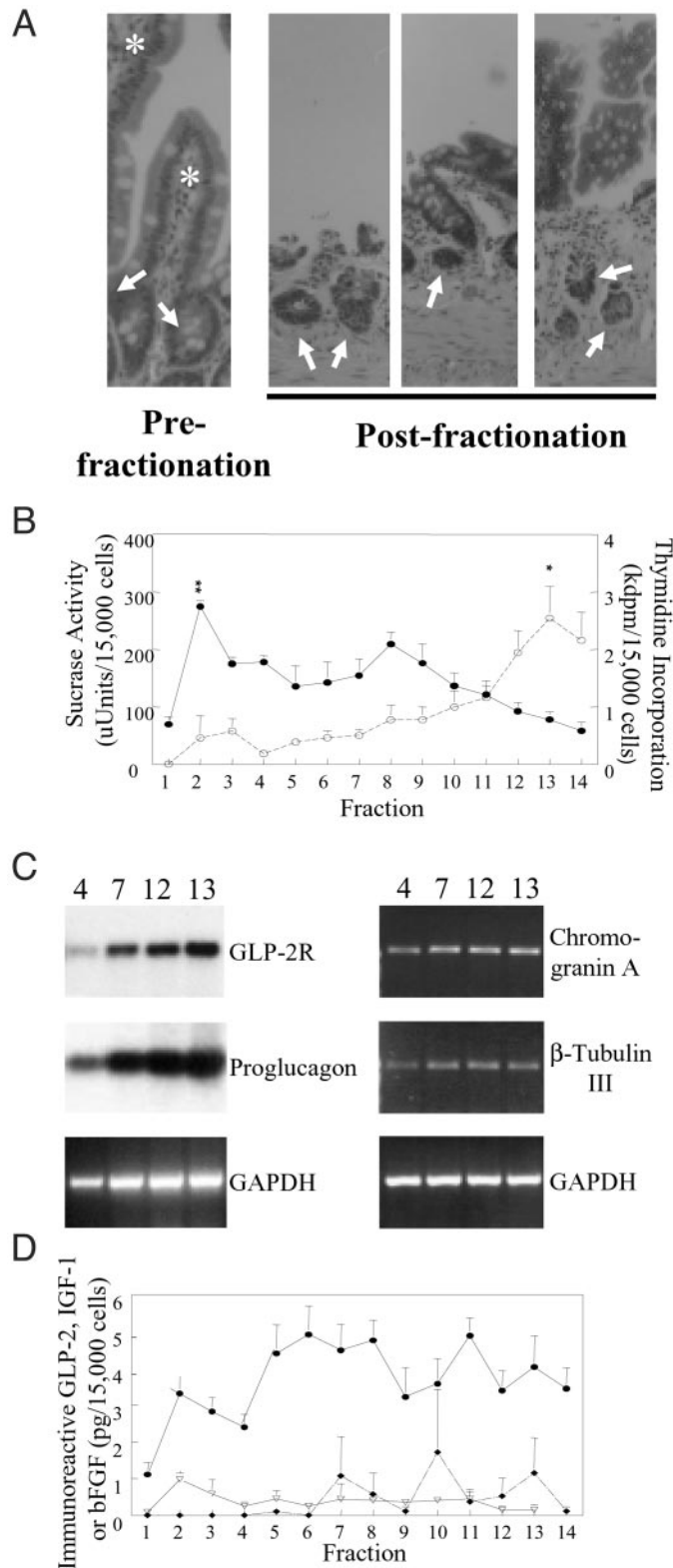
To investigate the characteristics of GLP-2R activation in the rat intestine, we developed a method for collection of primary rat small intestinal cells, using a calcium chelator in combination with mechanical dispersion to generate a series of 14 cell fractions. Morphometric analysis of the tissue fragments 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 identify crypt cells, which are located in the proliferative compartment of the small intestine (32), rats were injected *in vivo* with <sup>3</sup>H-thymidine 1 h before isolation of the cells. Analysis of the <sup>3</sup>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.

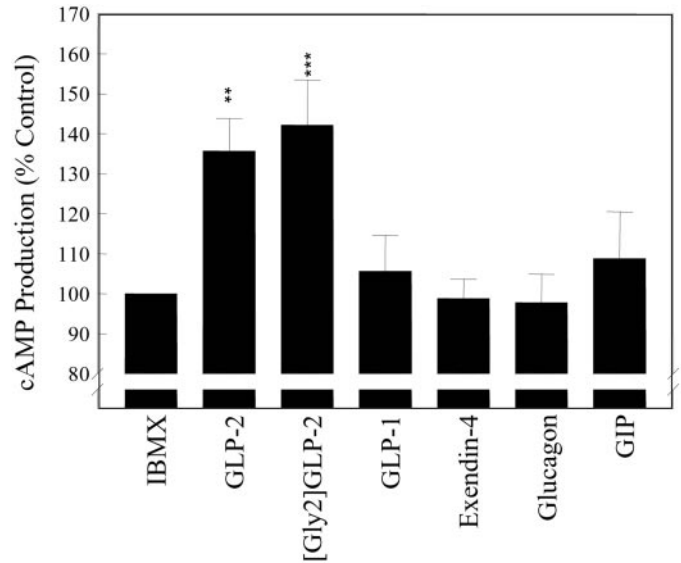
To study the effects of GLP-2 *in vitro*, we first determined whether GLP-2R mRNA transcripts were present in isolated rat mucosal cells by RT-PCR analysis of RNA from fractions 4, 7, 12, and 13. GLP-2R mRNA transcripts were detected in all fractions tested, as were transcripts for markers of both enteroendocrine cells (chromogranin A) and enteric neurons ( $\beta$ -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<sup>2</sup>]-GLP-2, stimulated a significant increase in cAMP levels ( $10^{-7}$  M each; to  $135 \pm 8\%$  and  $140 \pm 11\%$  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<sup>2</sup>]-GLP-2 ( $268 \pm 93\%$  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  $163 \pm 31\%$  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<sup>2</sup>]-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  $\mu$ M forskolin (positive control) or  $10^{-9}$  M [Gly<sup>2</sup>]-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<sup>2</sup>]-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<sup>2</sup>]-GLP-2 was completely abrogated by pretreatment with  $10^{-6}$  M [Gly<sup>2</sup>]-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 ( $\times 200$ ) 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  $^3\text{H}$ -thymidine levels (*dotted line*) in isolated rat small intestinal mucosal cells. Fourteen cell fractions were isolated and assayed for either sucrase ac-



**FIG. 2.** Specificity of the isolated rat small intestinal mucosal cell (fraction 12) response to GLP-2. All media contained  $100 \mu\text{M}$  IBMX. Cells ( $1.5 \times 10^4$ ) were incubated for 30 min with media alone or media +  $10^{-7} \text{M}$  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  $^3\text{H}$ -thymidine for 30 min (Fig. 4A). Addition of 10% fetal calf serum increased  $^3\text{H}$ -thymidine incorporation to  $162 \pm 8\%$  of control values ( $P < 0.001$ ), whereas  $[\text{Gly}^2]\text{-GLP-2}$  ( $10^{-9} \text{M}$ ) increased incorporation to  $128 \pm 8\%$  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  $^3\text{H}$ -thymidine incorporation ( $107 \pm 6\%$  of control;  $P < 0.05$  vs.  $[\text{Gly}^2]\text{-GLP-2}$  alone). In contrast, incubation of the cells with forskolin had no effect on  $^3\text{H}$ -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).  $[\text{Gly}^2]\text{-GLP-2}$  ( $10^{-9} \text{M}$ ) 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  $^3\text{H}$ -thymidine incorporation in a protein kinase A-dependent fashion, this was not associated with activation of p44/p42 MAPK.

tivity (determined as micrograms glucose liberated from sucrose;  $n = 4$ ) or  $[\text{H-methyl}]\text{thymidine}$  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 (kcpm: thousands of disintegrations per minute). **C**, RT-PCR of GLP-2R, proglucagon, chromogranin A,  $\beta$ -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-1, and bFGF content of isolated rat small intestinal mucosal cells. Fractions 1–14 were assayed for: GLP-2 by RIA ( $\bullet$ ;  $n = 6$ ); IGF-1 using an RIA kit ( $\nabla$ ;  $n = 3$ ); and bFGF using an EIA kit ( $\blacklozenge$ ;  $n = 4$ ).

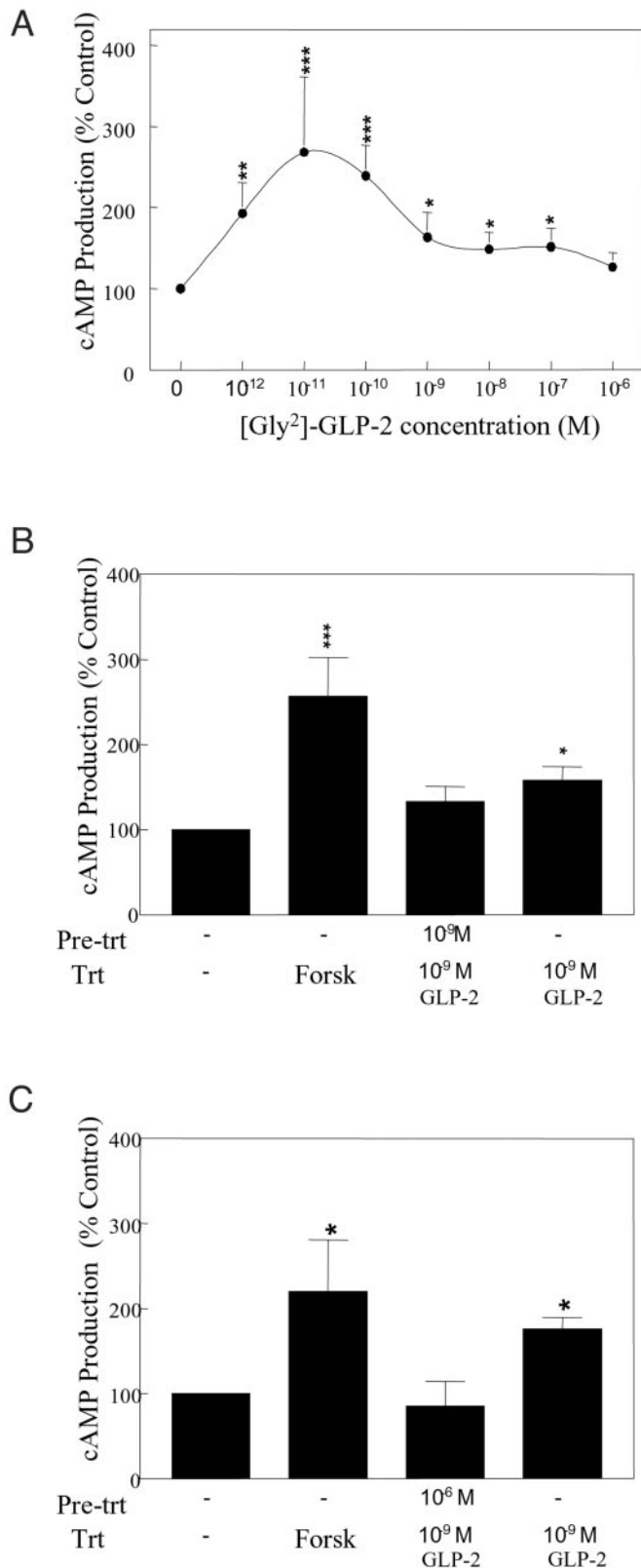


FIG. 3. Dose response and desensitization of isolated rat small intestinal mucosal cells (fraction 12) to GLP-2. All media contained 100  $\mu$ M IBMX. A, Cells ( $1.5 \times 10^4$ ) were incubated for 30 min with media alone or media +  $10^{-12}$ – $10^{-6}$  M human [Gly<sup>2</sup>]-GLP-2, and cAMP production was determined by RIA ( $n = 9$ ). B, Cells ( $1.5 \times 10^4$ ) were

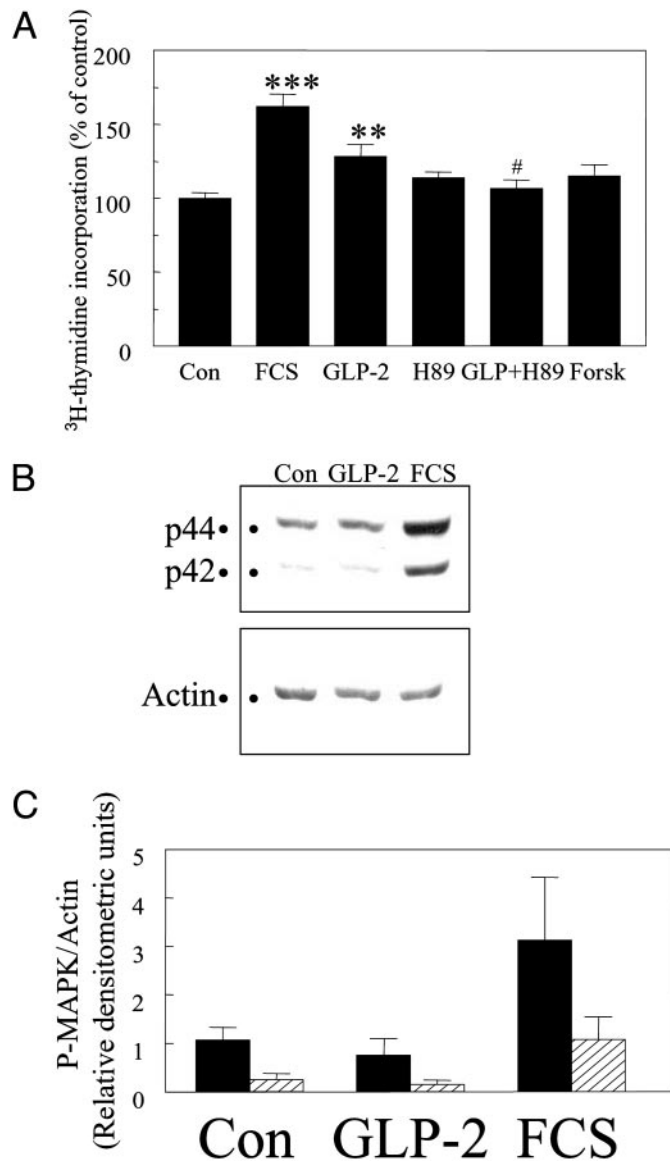
As previous studies have shown that the highly related GLP-1 and glucagon receptors exhibit dual signaling through both cAMP- and calcium-dependent pathways (35–37), we assessed whether GLP-2R signaling in the mucosal cells was coupled to changes in  $[Ca^{2+}]_c$ . Mucosal cells were preloaded with fura-2 AM ester and subjected to spectrofluorometric analysis in the presence or absence of GLP-2. [Gly<sup>2</sup>]-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  $\mu$ M), and this response was appropriately quenched by addition of MnCl<sub>2</sub> (6 mM). The responses to A23187 and MnCl<sub>2</sub> were used to calculate the resting  $[Ca^{2+}]_c$  of the rat mucosal cells as  $50 \pm 20$  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<sup>2</sup>]-GLP-2, and is maximally activated by  $10^{-9}$  to  $10^{-7}$  M [Gly<sup>2</sup>]-GLP-2 (17, 21, 38). By contrast, the endogenous rat GLP-2R studied here was maximally activated by [Gly<sup>2</sup>]-GLP-2 at  $10^{-11}$  M and responded less strongly to all doses of ligand between  $10^{-12}$  and  $10^{-7}$  M.

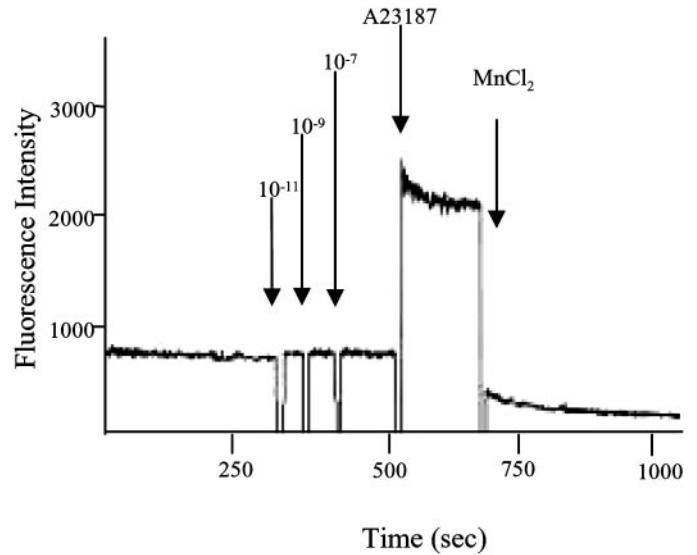
Similar differences have also been noted for activation of the GLP-1R by its ligand in cells expressing the endogenous receptor compared with heterologous cells transfected with the receptor [e.g. ED<sub>50</sub>s of 0.2 and 2 nM, respectively (20, 21)]. Such discrepancies in the dose-response profiles 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

pretreated (Pre-trl) for 60 min with media alone (–) or media +  $10^{-9}$  M human [Gly<sup>2</sup>]-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  $\mu$ M forskolin (Forsk, positive control), or media +  $10^{-9}$  M human [Gly<sup>2</sup>]-GLP-2 ( $n = 4$  each). C, Experiments were carried out as in (B), but with  $10^{-6}$  M human [Gly<sup>2</sup>]-GLP-2 in the Pre-trl ( $n = 4$  each). \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$  vs. control.



**FIG. 4.** Effects of GLP-2 on <sup>3</sup>H-thymidine incorporation and MAPK activation in rat small intestinal mucosal cells. **A**, Cells were preincubated with vehicle (Con) or 10  $\mu$ M H89 for 10 min, followed by incubation with <sup>3</sup>H-thymidine in vehicle, 10% fetal calf serum (FCS), 10<sup>-9</sup> M human [Gly<sup>2</sup>]-GLP-2  $\pm$  H89 or 100  $\mu$ M 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<sup>-9</sup> M [Gly<sup>2</sup>]-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).

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



**FIG. 5.** Cytosolic calcium in isolated rat small intestinal mucosal cells. Cells (10<sup>5</sup>) were suspended in phosphate-free HBSS containing 2.5 mM CaCl<sub>2</sub> and loaded with 2.5  $\mu$ M 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<sup>2</sup>]-GLP-2 to final concentrations of 10<sup>-11</sup>, 10<sup>-9</sup>, and 10<sup>-7</sup> M. Calcium ionophore A23187 (12.5  $\mu$ M) was used both as a positive control and for calibration. MnCl<sub>2</sub> (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.

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<sup>2</sup>]-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 [ $5 \times 10^{-11}$  M (29, 40)] and humans [10<sup>-12</sup> 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<sup>-6</sup> M [Gly<sup>2</sup>]-GLP-2 completely abrogated cAMP production in response to subsequent stimulation with [Gly<sup>2</sup>]-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  $\beta$ -cell line HIT-T15 have demonstrated that exposure to 10<sup>-7</sup> M GLP-1 followed by a short recovery period significantly reduces both cAMP accumulation and insulin secretion upon restimulation with 10<sup>-8</sup> 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 <sup>3</sup>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<sup>2</sup>]-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 <sup>3</sup>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.

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### References

- Xiao Q, Boushey R, Drucker DJ, Brubaker PL 1999 Secretion of the intestinotropic hormone glucagon-like peptide-2 is differentially regulated by nutrients in humans. *Gastroenterology* 117:99–105
- Rocca AS, Brubaker PL 1999 Role of the vagus nerve in mediating proximal nutrient-induced glucagon-like peptide-1 secretion. *Endocrinology* 140:1687–1694
- Hartmann B, Johnsen AH, Orskov C, Adelhorst K, Thim L, Holst JJ 2000 Structure, measurement, and secretion of human glucagon-like peptide-2. *Peptides* 21:73–80
- Dhanvantari S, Seidah NG, Brubaker PL 1996 Role of prohormone convertases in the tissue-specific processing of proglucagon. *Mol Endocrinol* 10:342–355
- Drucker DJ, Ehrlich P, Asa SL, Brubaker PL 1996 Induction of intestinal epithelial proliferation by glucagon-like peptide 2. *Proc Natl Acad Sci USA* 93:7911–7916
- Tsai CH, Hill M, Asa SL, Brubaker PL, Drucker DJ 1997 Intestinal growth-promoting properties of glucagon-like peptide-2 in mice. *Am J Physiol Endocrinol Metab* 273:E77–E84
- Brubaker PL, Izzo A, Hill M, Drucker DJ 1997 Intestinal function in mice with small bowel growth induced by glucagon-like peptide-2. *Am J Physiol Endocrinol Metab* 272:E1050–E1058
- Scott RB, Kirk D, MacNaughton WK, Meddings JB 1998 GLP-2 augments the adaptive response to massive intestinal resection in rat. *Am J Physiol Gastrointest Liver Physiol* 275:G911–G921
- Chance WT, Foley-Nelson T, Thomas I, Balasubramaniam A 1997 Prevention of parenteral nutrition-induced gut hypoplasia by coinfection of glucagon-like peptide-2. *Am J Physiol Gastrointest Liver Physiol* 273:G559–G563
- Drucker DJ, Yusta B, Boushey RP, DeForest L, Brubaker PL 1999 Human [Gly<sup>2</sup>]-GLP-2 reduces the severity of colonic injury in a murine model of experimental colitis. *Am J Physiol Gastrointest Liver Physiol* 276:G79–G91
- Schmidt PT, Hartmann B, Bregenholt S, Holst JJ, Claesson MH 2000 Deficiency of the intestinal growth factor, glucagon-like peptide 2, in the colon of SCID mice with inflammatory bowel disease induced by transplantation of CD4+T cells. *Scand J Gastroenterol* 35:522–527
- Boushey RP, Yusta B, Drucker DJ 1999 Glucagon-like peptide 2 decreases mortality and reduces the severity of indomethacin-induced murine enteritis. *Am J Physiol Endocrinol Metab* 277:E937–E947
- Boushey RP, Yusta B, Drucker DJ 2001 Glucagon-like peptide (GLP)-2 reduces chemotherapy-associated mortality and enhances cell survival in cells expressing a transfected GLP-2 receptor. *Cancer Res* 61:687–693
- Jeppesen PB, Hartmann B, Thulesen J, Graff J, Lohmann J, Hansen BS, Tofteng F, Poulsen SS, Madsen JL, Holst JJ, Mortensen PB 2001 Glucagon-like peptide 2 improves nutrient absorption and nutritional status in short-bowel patients with no colon. *Gastroenterology* 120:806–815
- Yusta B, Huang L, Munroe D, Wolff G, Fantasker R, Sharma S, Demchyshyn L, Asa SL, Drucker DJ 2000 Enterohormonal localization of GLP-2 receptor expression in humans and rodents. *Gastroenterology* 119:744–755
- Lund PK, Hoyt E, Simmons JG, Ulshen MH 1993 Regulation of intestinal glucagon gene expression during adaptive growth of small intestine. *Digestion* 54:371–373
- Yusta B, Somwar R, Wang F, Munroe D, Grinstein S, Klip A, Drucker DJ 1999 Identification of glucagon-like peptide-2 (GLP-2)-activated signaling pathways in baby hamster kidney fibroblasts expressing the rat GLP-2 receptor. *J Biol Chem* 274:30459–30467
- Munroe DG, Gupta AK, Kooshesh F, Vyas TB, Rizkalla G, Wang H, Demchyshyn L, Yang Z-J, Kamboj RK, Chen H, McCallum K, Sumner-Smith M, Drucker DJ, Crivici A 1999 Prototypic G protein-coupled receptor for the intestinotropic factor glucagon-like peptide 2. *Proc Natl Acad Sci USA* 96:1569–1573
- Brubaker PL, Drucker DJ 2002 Structure-function of the glucagon receptor superfamily of receptors: the glucagon, GIP, GLP-1 and GLP-2 receptors. *Receptors Channels* 8:179–188

20. Xiao Q, Giguere J, Parisien M, Jeng W, St-Pierre S, Brubaker PL, Wheeler MB 2001 Biological activities of glucagon-like peptide-1 analogs *in vitro* and *in vivo*. *Biochemistry* 40:2860–2869
21. Lovshin J, Estall J, Yusta B, Brown TJ, Drucker DJ 2001 Glucagon-like peptide (GLP)-2 action in the murine central nervous system is enhanced by elimination of GLP-1 receptor signaling. *J Biol Chem* 276:21489–21499
22. Bjerknes M, Cheng H 2001 Modulation of specific intestinal epithelial progenitors by enteric neurons. *Proc Natl Acad Sci USA* 98:12497–12502
23. Flint N, Cove FL, Evans GS 1991 A low temperature method for the isolation of small-intestinal epithelium along the crypt-villus axis. *Biochem J* 280:331–334
24. Chomczynski P, Sacchi N 1987 Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal Chem* 162:156–159
25. Heinrich G, Gros P, Lund PK, Bentley RC, Habener JF 1984 Pre-proglucagon messenger ribonucleic acid: nucleotide and encoded amino acid sequences of the rat pancreatic complementary deoxyribonucleic acid. *Endocrinology* 115:2176–2181
26. Piechaczyk M, Blanchard JM, Marty L, Dani C, Panabieres F, El-Sabouty S, Fort P, Jeanteur P 1984 Post-transcriptional regulation of glyceraldehyde-3-phosphate-dehydrogenase gene expression in rat tissues. *Nucleic Acids Res* 12:6951–6963
27. Lovshin J, Yusta B, Iliopoulos I, Migirdicyan A, Dableh L, Brubaker PL, Drucker DJ 2000 Ontogeny of the glucagon-like peptide-2 receptor axis in the developing rat intestine. *Endocrinology* 141:4194–4201
28. Dignas AU, Tsunekawa S, Podolsky DK 1994 Fibroblast growth factors modulate intestinal epithelial cell growth and migration. *Gastroenterology* 106:1254–1262
29. Brubaker PL, Crivici A, Izzo A, Ehrlich P, Tsai CH, Drucker DJ 1997 Circulating and tissue forms of the intestinal growth factor, glucagon-like peptide-2. *Endocrinology* 138:4837–4843
30. Drucker DJ, Shi Q, Crivici A, Sumner-Smith M, Tavares W, Hill M, DeForest L, Cooper S, Brubaker PL 1997 Regulation of the biological activity of glucagon-like peptide 2 *in vivo* by dipeptidyl peptidase IV. *Nat Biotechnol* 15:673–677
31. Gryniewicz G, Poenie M, Tsien RY 1985 A new generation of calcium indicators with greatly improved fluorescence properties. *J Biol Chem* 260:3440–3450
32. Pageot LP, Perreault N, Basora N, Francoeur C, Magny P, Beaulieu JF 2000 Human cell models to study small intestinal functions: recapitulation of the crypt-villus axis. *Microsc Res Tech* 49:394–406
33. Howarth GS, Shoubridge CA 2001 Enhancement of intestinal growth and repair by growth factors. *Curr Opin Pharmacol* 1:568–574
34. Fehmann HC, Habener JF 1991 Homologous desensitization of the insulinotropic glucagon-like peptide-1 (7–37) receptor on insulinoma (HIT-T15) cells. *Endocrinology* 128:2880–2888
35. Wheeler MB, Lu M, Dillon JS, Leng X-H, Chen C, Boyd AE, III 1993 Functional expression of the rat glucagon-like peptide-1 receptor, evidence for coupling to both adenylyl cyclase and phospholipase-C. *Endocrinology* 133:57–62
36. Yada T, Itoh K, Nakata M 1993 Glucagon-like peptide-1-(7–36)amide and a rise in cyclic adenosine 3', 5'-monophosphate increase cytosolic free Ca<sup>2+</sup> in rat pancreatic  $\beta$ -cells by enhancing Ca<sup>2+</sup> channel activity. *Endocrinology* 133:1685–1692
37. Hansen LH, Gromada J, Bouchelouche P, Whitmore T, Jelinek L, Kindsvogel W, Nishimura E 1998 Glucagon-mediated Ca<sup>2+</sup> signaling in BHK cells expressing cloned human glucagon receptors. *Am J Physiol Cell Physiol* 274:C1552–C1562
38. DaCabra MP, Yusta B, Sumner-Smith M, Crivici A, Drucker DJ, Brubaker PL 2000 Structural determinants for activity of glucagon-like peptide-2. *Biochem* 39:8888–8894
39. Fehmann HC, Pracht A, Göke B 1998 High-level expression of the GLP-1 receptor results in receptor desensitization. *Pancreas* 17:309–314
40. Fischer KD, Dhanvantari S, Drucker DJ, Brubaker PL 1997 Intestinal growth is associated with elevated levels of glucagon-like peptide 2 in diabetic rats. *Am J Physiol Endocrinol Metab* 273:E815–E820
41. Drucker DJ, Philippe J, Mojsos S, Chick WL, Habener JF 1987 Glucagon-like peptide I stimulates insulin gene expression and increases cyclic AMP levels in a rat islet cell line. *Proc Natl Acad Sci USA* 84:3434–3438
42. Widmann C, Dolci W, Thorens B 1996 Desensitization and phosphorylation of the glucagon-like peptide-1 (GLP-1) receptor by GLP-1 and 4-phorbol 12-myristate 13-acetate. *Mol Endocrinol* 10:62–75
43. Widmann C, Dolci W, Thorens B 1997 Internalization and homologous desensitization of the GLP-1 receptor depend on phosphorylation of the receptor carboxyl tail at the same three sites. *Mol Endocrinol* 11:1094–1102
44. Murphy GJ, Hruby VJ, Trivedi D, Wakelam JO, Houslay MD 1987 The rapid desensitization of glucagon-stimulated adenylyl cyclase is a cyclic AMP-independent process that can be mimicked by hormones which stimulate inositol phospholipid metabolism. *Biochem J* 243:39–46
45. Takasu H, Guo J, Bringham FR 1999 Dual signaling and ligand selectivity of the human PTH/PTHrP receptor. *J Bone Miner Res* 14:11–20
46. Krsmanovic LZ, Mores N, Navarro CE, Arora KK, Catt KJ 2003 An agonist-induced switch in G protein coupling of the gonadotropin-releasing hormone receptor regulates pulsatile neuropeptide secretion. *Proc Natl Acad Sci USA* 100:2969–2974