

Regulation of Glucagon-Like Peptide-1 Synthesis and Secretion in the GLUTag Enteroendocrine Cell Line*

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ABSTRACT

Glucagon-like peptide-1 (GLP-1) released from the intestine is a potent stimulator of glucose-dependent insulin secretion. To elucidate the factors regulating GLP-1 secretion, we have studied the enteroendocrine GLUTag cell line. GLP-1 secretion was stimulated in a dose-dependent fashion by activation of protein kinase A or C with forskolin or phorbol 12,13-dibutyrate, respectively (by 2.3 ± 0.5 -fold at $100 \mu\text{M}$ and 4.3 ± 0.6 -fold at $0.3 \mu\text{M}$, respectively; $P < 0.01$ – 0.001). Of the regulatory peptides tested, only glucose-dependent insulinotropic peptide stimulated the release of GLP-1 (by 2.3 ± 0.2 -fold at $0.1 \mu\text{M}$; $P < 0.001$); glucagon was without effect, and paradoxically, the inhibitory neuropeptide somatostatin-14 increased secretion slightly (by 1.6 ± 0.3 -fold at $0.01 \mu\text{M}$; $P < 0.05$). In tests of several neurotransmitters, only the cholinergic agonists carbachol and bethanechol stimulated peptide secretion in a dose-dependent fashion (by $2.3 \pm$

0.5 - and 1.7 ± 0.3 -fold at $1000 \mu\text{M}$; $P < 0.05$ – 0.001); the β -adrenergic agonist isoproterenol and the chloride channel inhibitor γ -aminobutyric acid did not affect release of GLP-1. Long chain monounsaturated fatty acids (18:1), but not saturated fatty acids (16:0), also stimulated the release of GLP-1 (by 1.7 ± 0.1 -fold at $150 \mu\text{M}$; $P < 0.001$). Consistent with the presence of a cAMP response element in the proglucagon gene, activation of the protein kinase A-dependent pathway with forskolin increased proglucagon messenger RNA transcript levels by 2-fold ($P < 0.05$); glucose-dependent insulinotropic peptide and phorbol 12,13-dibutyrate were without effect. Therefore, by comparison with results obtained using primary L cell cultures or *in vivo* models, GLUTag cells appear to respond appropriately to the regulatory mechanisms controlling intestinal GLP-1 secretion. (*Endocrinology* 139: 4108–4114, 1998)

GLUCAGON-LIKE peptide-1 (GLP-1) is a potent stimulator of glucose-dependent insulin secretion. Administration of GLP-1 to patients with type II diabetes normalizes both fasting and postprandial glycemia (1–13), not only through stimulation of insulin release, but also through concomitant inhibition of glucagon secretion (5, 9, 11, 13) and gastric motility (5, 14) and, possibly, enhancement of insulin sensitivity (8, 15, 16). GLP-1 is normally synthesized and secreted by the intestinal L cell (17–21); thus, stimulation of endogenous secretion represents an alternative approach to increasing levels of GLP-1 in type II diabetes. It is therefore essential that the factors regulating GLP-1 release from the L cell be elucidated.

A number of *in vitro* cell culture systems have been developed as models of the intestinal L cell, each of which has both advantages and drawbacks. For example, fetal rat intestinal cell (FRIC) cultures are heterogeneous in their cell population, although they have proven to be an excellent model of the rat L cell otherwise, releasing GLP-1 in response to a wide variety of different signal transduction pathways and extracellular mediators (18, 20–24). FRIC cells have also been used for studies of changes in proglucagon messenger RNA (mRNA) transcript levels (18), although their heterogeneity has proven to be limiting for more detailed molecular

analyses of proglucagon gene expression. An alternative model, the isolated canine L cell (25–27), provides a more homogeneous cell population, but this culture system requires centrifugal elutriation to prepare and is therefore relatively expensive. A secretin tumor cell line (STC-1) that secretes GLP-1 has also been used as an L cell model (28); however, STC-1 cells are poorly differentiated and multipotential. Hence, the similarity of this intestinal S cell model to the GLP-1-producing L cell is unclear.

The paucity of L cell models encouraged us to develop an L cell line (GLUTag) from intestinal endocrine tumors arising in the large bowel in proglucagon-simian virus 40 large T antigen transgenic mice (29). GLUTag cells express the proglucagon gene at high levels and process proglucagon to a number of proglucagon-derived peptides, including GLP-1. GLUTag cells have been demonstrated to secrete GLP-1 in response to intracellular stimulators of the protein kinase A (PKA) and protein kinase C (PKC) pathways, such as forskolin and phorbol esters, respectively (29). As FRIC cultures also exhibit regulated GLP-1 secretion in response to activation of PKA- and PKC-dependent pathways (18, 20–24), we hypothesized that the GLUTag cell line would be a good model for further studies of GLP-1 release in response to a variety of potential secretagogues.

Materials and Methods

Reagents

Forskolin, isobutylmethylxanthine (IBMX), phorbol 12,13-dibutyrate (PDBU), α -ketoisocaproic acid (KIC), glucose-dependent insulinotropic peptide (GIP), glucagon, isoproterenol, somatostatin-14 (S14), carbachol, and γ -aminobutyric acid (GABA) were gifts from Eli Lilly Co. (India-

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napolis, IN). Bethanechol was purchased from Merck Frosst Canada (Kirkland, Canada), and tissue culture reagents were obtained from Life Technologies (Burlington, Canada). Oleic (18:1) and palmitic (16:0) acids and phorbol 12-myristate-13-acetate (PMA) were obtained from Sigma Chemical Co. (St. Louis, MO). To enhance fatty acid solubility (30, 31), stock solutions were prepared in dimethylsulfoxide and then diluted 1:1000 into serum (albumin)-containing tissue culture medium.

Cell culture

GLUTag cells were grown in DMEM (low glucose) containing 10% (vol/vol) FBS, as previously described (29). The medium was changed every 3–4 days. Cells were then trypsinized, plated in 24-well cultures plates, and allowed to reach 60–80% confluence. On the day of the experiment, cells were washed twice with HBSS and incubated with test agents in DMEM containing 0.5% (vol/vol) FBS for 2 h. Each experiment was repeated on a minimum of two different occasions to make at least $n = 4$.

FRIC cultures were prepared from term fetal rat intestines, as previously described (18, 20–24). In brief, intestines from one litter of rats were pooled to make $n = 1$, and the cells were dispersed by incubation with collagenase (40 mg/dl; SigmaBlend Type H, Sigma Chemical Co.), hyaluronidase (40 mg/dl; type II, Sigma Chemical Co.), and deoxyribonuclease I (5 mg/dl; Sigma Chemical Co.). Cells were plated at a density of 0.6 fetal rat intestines/60-mm dish in DMEM (high glucose) containing 5% (vol/vol) FBS, 50 IU/ml penicillin, and 50 μ g/ml streptomycin and were allowed to recover overnight. Cells were then washed with HBSS and incubated for 2 h with test agents in DMEM (low glucose) containing penicillin, streptomycin, and 20 μ U/ml insulin.

Peptide extraction and analysis

At the end of the incubation period, medium was collected, centrifuged to remove any floating cells, and made to 0.1% (vol/vol) with trifluoroacetic acid. Cells were homogenized in 1 N HCl containing 5% (vol/vol) HCOOH, 1% (vol/vol) trifluoroacetic acid, and 1% (vol/vol) NaCl. Peptides and small proteins were then extracted from cells and cell medium by passage through a cartridge of C_{18} silica (Sep-Pak, Waters Associates, Milford, MA). We have previously reported that this methodology affords a greater than 88% recovery of intact proglucagon-derived peptides (PGDPs) (22, 23). Samples were subjected to RIA for GLP-1-(36)NH₂ using the GLP-1-(7–36)NH₂ antiserum from Affinity Research (Nottingham, UK) that recognizes GLP-1(7–36)NH₂ and GLP-1-(1–36)NH₂ equally. This antiserum does not cross-react with Gly-extended forms of GLP-1 (19, 32). Previous studies have demonstrated that GLP-1-(7–36)NH₂ is the predominant form of GLP-1 synthesized by GLUTag cells (29).

We have previously demonstrated that GLP-1 secretion by FRIC cultures occurs in parallel with that of other PGDPs, most notably glucagon-like immunoreactivity (GLI) (20, 21). Therefore, as in previous studies (18, 20–24), FRIC cultures were subjected to RIA for changes in GLI secretion using antiserum K4023 (Biospecific, Emeryville, CA).

RNA extraction and analysis

Cells were incubated with test agents for 12 h, after which total cellular RNA was isolated by the guanidium-isothiocyanate method (33). Total RNA was subjected to electrophoresis using a 1% (wt/vol) agarose-formaldehyde gel, and the gel was stained with ethidium bromide to assess the migration and integrity of the RNA. RNA was then transferred onto a nylon membrane, fixed by exposure to UV light, and hybridized using a full-length complementary DNA (cDNA) probe for proglucagon, as previously described (29). To control for loading and transfer efficiency, the blots were rehybridized with a cDNA for 18S ribosomal RNA.

Data analysis

Secretion was calculated as the percentage of the total cell content of peptide (GLP-1 or GLI) that was found in the medium [$100 \times$ medium peptide/(medium + cell peptide)]. The total content of GLP-1 in control GLUTag cultures (medium plus cells) was 1.9 ± 0.2 ng/well ($n = 10$), and this was not altered by treatment with high doses of any of the test

agents. Under control conditions, $8.5 \pm 0.8\%$ ($n = 10$) of the total cell content was released into the medium during the 2-h incubation period. Statistical differences were determined by ANOVA using $n-1$ custom hypotheses tests on an SAS program (Statistical Analysis Systems, Cary, NC) for IBM computers.

Results

Activation of PKA- and/or PKC-dependent pathways is known to stimulate intestinal PGDP secretion from FRIC cultures (18, 20, 21, 23) and isolated canine L cells (25, 26). Activation of the PKA-dependent pathway with forskolin also stimulated dose-dependent increases in the release of GLP-1 from GLUTag cells (Fig. 1). The forskolin response was linear within the dose range tested, reaching 2.3 ± 0.5 -fold of control values at 100 μ M ($P < 0.01$). Treatment of GLUTag cells with the cAMP phosphodiesterase inhibitor, IBMX, similarly increased GLP-1 release, to a maximum of $186 \pm 37\%$ of the control value ($P < 0.05$) at 10 μ M (Fig. 1). GLP-1 secretion was also stimulated by treatment with PDBU; the response to this phorbol ester was highly significant, reaching $426 \pm 61\%$ of the control value at 0.3 μ M ($P < 0.001$). Treatment with a second phorbol ester, PMA (1 μ M), also increased peptide release, to 1.7 ± 0.2 -fold of the control value ($P < 0.001$), whereas down-regulation of PKC via 24-h pretreatment with PMA completely abrogated this response ($91 \pm 9\%$ of the control value; Fig. 1). In contrast to the PKA and PKC pathways, incubation with KIC decreased the release of GLP-1 in a biphasic fashion, with inhibition occurring at low (10–30 μ M; $P < 0.05$ –0.01), but not higher (100–300 μ M), concentrations. KIC is an amino acid metabolite that has been reported to stimulate phospholipase C in islet cells (34). As the effect of KIC on FRIC cultures has not previously been reported, a similar dose-response curve was tested in this system (Fig. 2). KIC had no effect on PGDP secretion by FRIC cultures, although the positive control (forskolin plus IBMX) indicated that the cells were responsive to secretagogues.

The intestinal L cell is known to be modulated by a variety of hormones in the FRIC culture model (24). Thus, to assess the effects of regulatory peptides on GLP-1 secretion, GLUTag cells were incubated with GIP, glucagon, or S14 (Fig. 3). GIP increased GLP-1 secretion in a dose-dependent fashion, with a significant increment to $227 \pm 24\%$ of the control value at 0.1 μ M ($P < 0.001$). GLUTag cells were unresponsive to the structurally related peptide, glucagon, at all doses tested; however, GLP-1 release was increased in response to treatment with the highest dose of S14 (0.01 μ M; $P < 0.05$).

Treatment of GLUTag cells with the cholinergic agonist carbachol induced GLP-1 release at concentrations of 500–1000 μ M ($P < 0.05$ –0.001), but not at lower doses (Fig. 4). As FRIC cultures have previously been found to be sensitive to the muscarinic agonist bethanechol (24), this agent was also tested with GLUTag cells and was found to stimulate GLP-1 release in the same dose range as carbachol ($P < 0.05$). Although the incremental response to 1000 μ M bethanechol appeared to be reduced compared with that for 1000 μ M carbachol, this difference did not reach statistical significance. GLP-1 secretion was not altered by treatment of GLUTag cells with either isoproterenol or GABA. Studies in

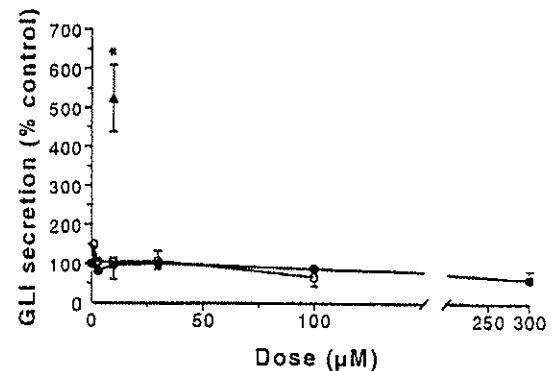
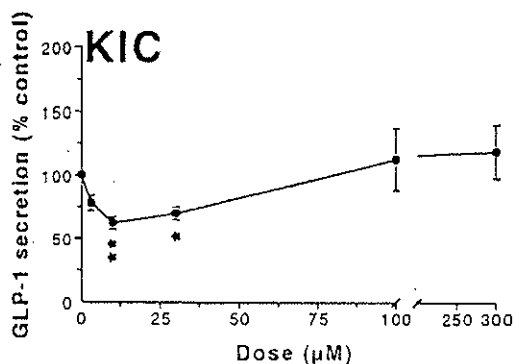
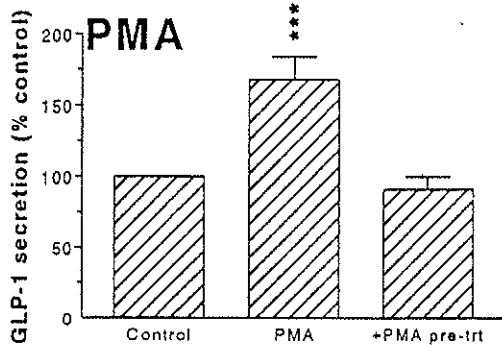
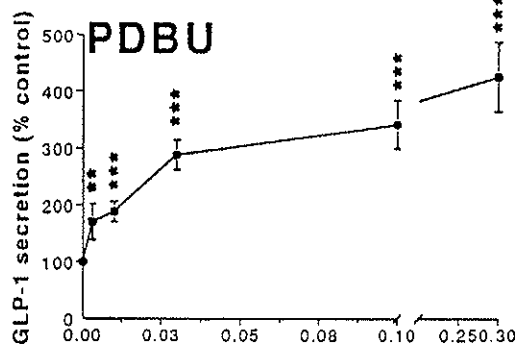
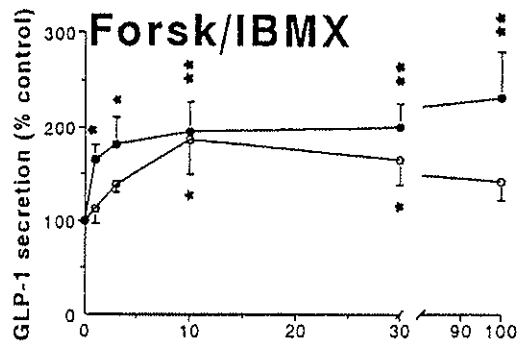


FIG. 2. Secretion of GLI by FRIC cultures in response to incubation for 2 h with forskolin plus IBMX (triangle; 10 μ M each) or different doses of KIC (closed circles) or GABA (open circles; $n = 4$ each). Secretion into the medium was normalized to the total cell content of the culture well and is expressed as a percentage of the control value. *, $P < 0.05$ vs. paired controls.

FRIC cultures similarly indicated a lack of effect of GABA on the L cell (Fig. 2).

FRIC cultures have previously been shown to secrete GLP-1 in response to long chain monounsaturated, but not long chain saturated, fatty acids (21). GLUTag cells were therefore also tested for responsiveness to oleic (18:1) and palmitic (16:0) acids (Fig. 5). Treatment with the monounsaturated fatty acid stimulated GLP-1 secretion by 1.7 ± 0.1 -fold ($P < 0.001$) at a dose of 150 μ M, whereas the saturated fatty acid appeared to have either a slight inhibitory or no effect.

To determine whether physiological agents that stimulated GLP-1 secretion were also coupled to activation of proglucagon gene expression, GLUTag cells were incubated for 12 h in the presence of 0.1 μ M GIP, after which total cellular RNA was isolated for Northern analysis. The results of this experiment demonstrated that, despite the stimulatory effects of GIP on GLP-1 secretion in the same experiment, this peptide did not stimulate proglucagon gene expression (Fig. 6). In paired control experiments, forskolin/IBMX treatment increased the levels of proglucagon mRNA transcripts (by 2-fold; $P < 0.05$), whereas PDBU was without effect.

Discussion

Previous *in vitro* studies on GLP-1 secretion have been restricted in their scope due to various limitations of the available systems. The development of the enteroendocrine GLUTag cell line has now permitted analysis of this system as a model of the intestinal L cell. Release of GLP-1 by GLUTag cells was increased by intracellular activation of both PKA-dependent (with forskolin or IBMX) and PKC-

FIG. 1. Secretion of GLP-1 by GLUTag cells in response to incubation for 2 h with different doses of forskolin (closed circles; $n = 7$), IBMX (open circles; $n = 5$), PDBU ($n = 7$), or KIC ($n = 6$). Cells treated with 1 μ M PMA ($n = 8$) were exposed to either culture medium alone or to 1 μ M PMA (pretreatment) for 24 h before the experimental period. Secretion into the medium was normalized to the total cell content of the culture well and is expressed as a percentage of the control value. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ (vs. paired controls).

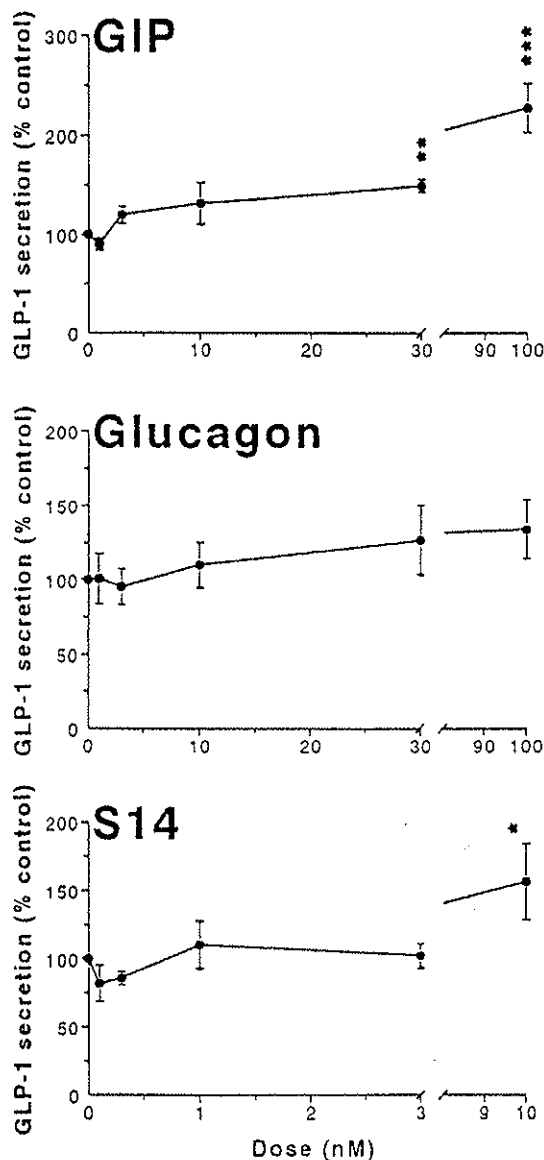


Fig. 3. Secretion of GLP-1 by GLUTag cells in response to incubation for 2 h with different doses of GIP ($n = 5$), glucagon ($n = 4$), or S14 ($n = 6$). Secretion into the medium was normalized to the total cell content of the culture well and is expressed as a percentage of the control value. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ (vs. paired controls).

dependent (with PDBU or PMA) pathways. Similar studies in both FRIC (18, 20, 21, 23) and isolated canine L cell (25, 26) cultures indicated the importance of these pathways in the regulation of intestinal PGDP secretion. The results of the present study extend these findings by the demonstration that treatment with the potential phospholipase C activator, KIC, does not enhance PGDP secretion by either FRIC or GLUTag cells and, indeed, may actually inhibit secretion in this cell line. It must be noted, however, that previous studies with KIC in islet cells used doses 100-fold greater than those used in the present study (34). Thus, a stimulatory effect of KIC at such high concentrations cannot presently be precluded. Nonetheless, the results of the present study indicate

that major intracellular pathways determining GLP-1 secretion by the L cell appear to be linked to PKA and PKC.

Several regulatory peptides that activate PKA-dependent pathways through the seven-transmembrane domain, G protein-linked receptors were tested for their effects on GLP-1 release by GLUTag cells, including GIP (35) and glucagon (36). Of these, only GIP was found to stimulate GLP-1 release; glucagon was without effect on the GLUTag cells, consistent with the results of studies using FRIC cultures (24). Studies using models as diverse as FRIC cultures (20, 24), isolated perfused rat ileum (37, 38), and the anesthetized rat (39), have all indicated that GIP can stimulate secretion by the rat intestinal L cell. Interestingly, this effect appears to be species specific, as the human L cell is not stimulated by GIP *in vivo* (1, 4). Very recently, we demonstrated that the effects of physiological concentrations of GIP on the rat L cell *in vivo* are exerted indirectly, through the vagus nerve (40). At supraphysiological concentrations, however, the effects of GIP on the L cell are not prevented by vagotomy. Thus, our finding that the GLUTag cells are responsive to GIP at relative high concentrations only (0.03–0.1 μM) is consistent with the *in vivo* sensitivity of the L cell to this peptide.

In keeping with a role for the vagus in modulating GLP-1 release in the rat *in vivo*, both carbachol and bethanechol were found to stimulate peptide secretion by GLUTag cells at doses of 500–1000 μM . The dose-response curve for these effects was identical to that of a previous study using bethanechol with FRIC cultures (24). However, in the perfused rat ileum model, cholinergic agonists stimulate the release of GLP-1 at substantially lower concentrations (10–100 μM) (37, 38). The reasons for the differences between the two *in vitro* models and the *in situ* setting are not clear; however, the possible involvement of other mediators cannot be discounted in the perfused rat ileum model. Nonetheless, a cholinergic pathway does appear to be important for GLP-1 secretion in humans, as atropine treatment prevents GLP-1 secretion during an oral glucose tolerance test (41). Interestingly, this effect is also species specific, as carbachol is actually inhibitory to the canine L cell *in vitro* (25, 26). Finally, as bethanechol acts through muscarinic receptors only, whereas carbachol binds to both muscarinic and nicotinic receptors, the present findings suggest a role for a muscarinic receptor in the regulation of GLP-1 secretion. Although the secretin tumor (STC-1) cell line is not a true model of the L cell, studies using these cells have indicated an involvement of the M3 receptor subtype in modulating GLP-1 release (28). Further investigations to determine the receptor subtype expressed by the GLUTag cells are therefore clearly warranted.

In contrast to the stimulatory effects of muscarinic agonists on both FRIC cultures and GLUTag cells, other neuromodulators, including the β -adrenergic agonist isoproterenol and the chloride channel inhibitor GABA, had no effect on peptide secretion in these culture systems (present data and Ref. 24). These findings are consistent with those reported for the perfused rat ileum (37, 38). However, as for the regulation by other secretagogues, the lack of effect of the β -adrenergic agonist is species dependent, as it has been reported that the canine L cell is stimulated by epinephrine (25, 26).

The intestinal neuropeptide S14 is a known inhibitor of the L cell in FRIC cultures (24), canine L cells *in vitro* (25–27), and

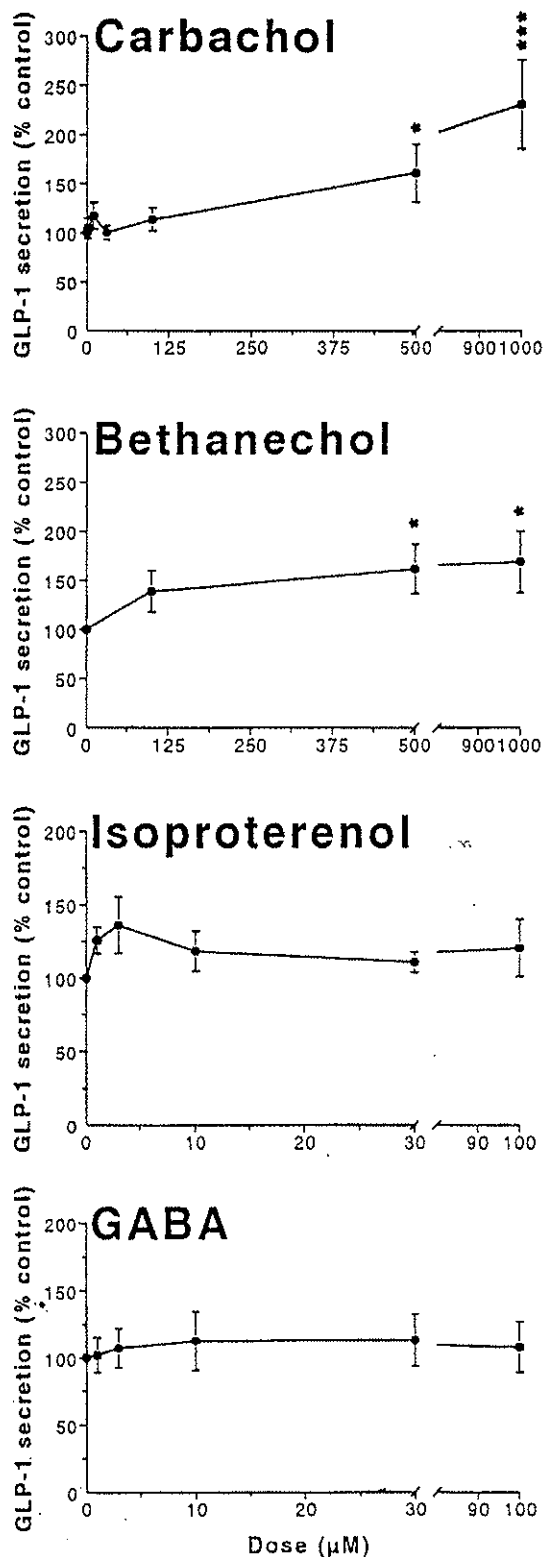


FIG. 4. Secretion of GLP-1 by GLUTag cells in response to incubation for 2 h with different doses of carbachol ($n = 7$ for 1–100 μM ; $n = 3$ for 500–1000 μM), bethanechol ($n = 3$), isoproterenol ($n = 8$), or GABA ($n = 6$). Secretion into the medium was normalized to the total cell content of the culture well and is expressed as a percentage of the control value. *, $P < 0.05$; ***, $P < 0.001$ (vs. paired controls).

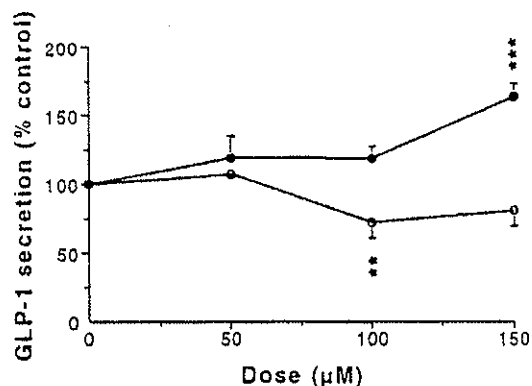


FIG. 5. Secretion of GLP-1 by GLUTag cells in response to incubation for 2 h with different doses of oleic (18:1) or palmitic (16:0) acid ($n = 4-8$). Secretion into the medium was normalized to the total cell content of the culture well and is expressed as a percentage of the control value. **, $P < 0.01$; ***, $P < 0.001$ (vs. paired controls).

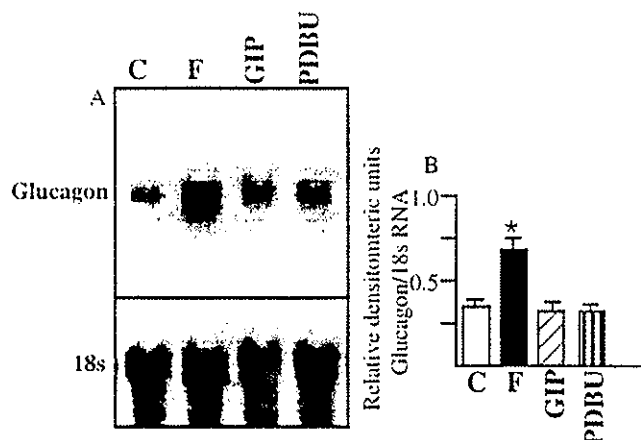


FIG. 6. mRNA transcript levels after incubation of GLUTag cells with medium alone (control; C), forskolin plus IBMX (10 μM each; F), GIP (0.1 μM), or PDBU (0.3 μM) for 12 h. The Northern blot was sequentially hybridized with cDNA probes for proglucagon and 18S mRNAs. A representative Northern blot is shown (A), as are the integrated results for three experiments (B; plotted as values for proglucagon mRNA normalized to the signal obtained for 18S RNA). *, $P < 0.05$ (vs. paired controls).

both rats and dogs *in vivo* (42, 43). Studies in FRIC cultures (24) have suggested the presence of SSTR5, a somatostatin-28-prefering subtype on the L cell, rather than SSTR2, the S14-prefering receptor (44). The results of the present study suggest that neither of these receptors is present on GLUTag cells, as both should be associated with decreased GLP-1 release through inhibition of the PKA pathway. Although it remains to be established why GLP-1 secretion by the GLUTag cells was actually increased by treatment with high doses of S14, this finding is not without precedent, as stimulatory effects of somatostatin have been observed in the neuroendocrine GH₄C₁ cells via release of the G $\beta\gamma$ -subunit from inhibitory G α proteins (45, 46).

Finally, GLP-1 secretion was also found to be stimulated by a long chain monounsaturated fatty acid (18:1), but not by a similar length saturated fatty acid (16:0), consistent with our previous results using FRIC cultures (21, 24). Oleic acid has also been reported to stimulate the canine L cell *in vitro*

over a similar dose range (27). These findings are consistent with several reports of stimulation of the rat ileal L cell *in vivo* by mixed fats (39) and therefore suggest that diets enriched in long chain monounsaturated fatty acids, such as olive oil, may be a useful approach to enhance the release of GLP-1 *in vivo*. A summary of the similarities and differences between the secretory responses of the primary rat L cell in culture (e.g. FRIC cultures) and the GLUTag cell line is shown in Table 1.

Despite evidence for secretory activity of GIP in the GLUTag cell system, activation of the PKA-dependent signal transduction pathway for 12 h with GIP did not increase proglucagon mRNA transcript levels in GLUTag cells. These findings were somewhat unexpected, as we previously reported that treatment of FRIC cultures for 24 h with GIP increases total GLP-1 levels slightly, but significantly (20). In control experiments, however, proglucagon mRNA transcript levels were increased by forskolin/IBMX-induced activation of PKA-dependent pathways, consistent with previous findings in both FRIC cultures and GLUTag cells using forskolin/IBMX (18, 29) and with nuclear run-on studies in GLUTag cells showing activation of proglucagon gene transcription by forskolin/IBMX, presumably through the proglucagon gene cAMP response element (29). The reasons for the apparent lack of effect of GIP are thus not clear, but may relate to the duration of treatment (12 vs. 24 h), the degree of activation of the PKA pathway by each agent, and/or possible translational vs. transcriptional effects. Finally, indirect evidence using a luciferase reporter gene linked to proglucagon promoter sequences has suggested that PKC may activate proglucagon gene transcription in the α TC2 islet cell line (47). However, the results of the present study indicate a total lack of effect of PDBU on proglucagon mRNA transcript levels in the enteroendocrine GLUTag cell line. We have similarly reported that activation of the PKC-dependent pathway in GLUTag cells with cholecystokinin (29) or in FRIC cultures with PMA (18) does not augment total PGDP or proglucagon mRNA transcript levels. Taken together, therefore, these observations highlight the importance of the cAMP-dependent pathway in the regulation of both proglucagon gene expression and GLP-1 secretion by the intestinal L cell.

The results of the present study indicate that the GLUTag cells appear to represent a good model of the intestinal L cell;

they exhibit appropriate responses to known stimulators of GLP-1 secretion and are unaffected by factors that do not modulate GLP-1 release in other systems. Thus, these cells should be useful for further studies to evaluate potential GLP-1 secretagogues.

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TABLE 1. Comparison of secretory responses of FRIC cultures and GLUTag cells

Treatment	FRIC response	GLUTag response
PKA activation	↑	↑
PKC activation	↑	↑
Phospholipase C activation	↔	↔
GIP	↑	↑
Glucagon	↔	↔
S14	↓	(↑)
Muscarinic agonists	↑	↑
β-Adrenergic agonist	↔	↔
GABA	↔	↔
Fatty acids (18:1)	↑	↑
Fatty acids (16:0)	↔	↔

↑, Stimulation; ↓, inhibition; ↔, no effect.

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