Bombesin and nutrients independently and additively regulate hormone release from GIP/Ins cells

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Submitted 30 July 2004; accepted in final form 17 September 2004

Li, Lin, and Burton M. Wice. Bombesin and nutrients independently and additively regulate hormone release from GIP/Ins cells. Am J Physiol Endocrinol Metab 288: E208–E215, 2005. First published September 21, 2004; doi:10.1152/ajpendo.00346.2004.—Glucose-dependent insulino tropic polypeptide (GIP) regulates glucose homeostasis and high-fat diet-induced obesity and insulin resistance. Therefore, elucidating the mechanisms that regulate GIP release is important. GIP is produced by K cells, a specific subtype of small intestinal enteroendocrine (EE) cell. Bombesin-like peptides produced by enteric neurons and luminal nutrients stimulate GIP release in vivo. We previously showed that PMA, bombesin, meat hydrolysat e, glycerolal dehyde, and methylpyruvate increase hormone release from a GIP-producing EE cell line (GIP/Ins cells). Here we demonstrate that bombesin and nutrients additively stimulate hormone release from GIP/Ins cells. In various cell systems, bombesin and PMA regulate cell physiology by activating PKD signaling in a PKC-dependent fashion, whereas nutrients regulate cell physiology by inhibiting AMPK signaling. Western blot analyses of GIP/Ins cells using antibodies specific for activated and/or phosphorylated forms of PKD and AMPK and one substrate for each kinase revealed that bombesin and PMA, but not nutrients, activated PKC, but not PKD. Conversely, nutrients, but not bombesin or PMA, inhibited AMPK activity. Pharmacological studies showed that PKC inhibition blocked bombesin- and PMA-stimulated hormone release, but AMPK activation failed to suppress nutrient-stimulated hormone secretion. Forced expression of constitutively active vs. dominant negative PKDs or AMPKs failed to perturb bombesin- or nutrient-stimulated hormone release. Thus, in GIP/Ins cells, PKC regulates bombesin-stimulated hormone release, whereas nutrients may control hormone release by regulating the activity of AMPK-related kinases, rather than AMPK itself. These results strongly suggest that K cells in vivo independently respond to neuronal vs. nutritional stimuli via two distinct signaling pathways.

K cells; protein kinase C; protein kinase D; adenosine 5’-monophosphate-activated protein kinase; adenosine 5’-monophosphate-activated protein kinase-related kinase; enteric neurons; nutrient sensing

ENTEROENDOCRINE (EE) CELLS are hormone-producing intestinal epithelial cells. Although these singly dispersed cells comprise <1% of the intestinal epithelium, as a whole they represent the largest endocrine organ in the body. There are at least 16 different subpopulations of EE cells, based on the major product(s) synthesized and secreted by individual cells (1). These hormones play important roles in regulating gastrointestinal secretion, motility, and blood flow and also regulate whole animal physiology (1, 35, 43, 48, 50, 59). For example, glucagon-like peptides-1 and -2 are important growth and trophic factors for islet β-cells and the intestine, respectively (13). Glucose-dependent insulino tropic polypeptide (GIP), ghrelin, CCK, and peptide tyrosine regulate food intake and/or adiposity (3, 5, 40, 49, 57). GIP and glucagon-like peptide-1 potentiate glucose-stimulated insulin release and thus play important roles in maintaining blood glucose homeostasis (18). Surprisingly, little is known about the molecular mechanisms that regulate hormone release from different subtypes of EE cells. Because GIP promotes both obesity and glucose-stimulated insulin release, we have been particularly interested in understanding the molecular mechanisms that regulate release of this hormone.

GIP is secreted in response to nutrients present in the lumen of the gut but not those circulating in the blood (12, 18, 48). GIP release is also regulated by molecules produced by enteric neurons [e.g., bombesin-like peptides (29)], other enteroendocrine cells [e.g., somatostatin inhibits GIP release (59)], and possibly enterocytes (42, 54, 60). Therefore, K cells integrate input from numerous sources to release appropriate amounts of GIP. Using GIP/Ins cells (42), we have begun to study the regulation of K cell physiology using the well-characterized islet β-cell as a model.

In β-cells, glucose metabolism increases the intracellular ATP-to-ADP (ATP/ADP) ratio, which, in turn, inhibits ATP-sensitive K+ (KATP) channels. This causes cell depolarization, influx of calcium via voltage-dependent calcium channels, and finally exocytosis of insulin from secretory granules. Islet β-cells also exhibit KATP channel-independent mechanisms of secretion that involve mobilization of calcium from endoplasmic reticulum (ER)-derived stores (44). Release of ER calcium stores can be regulated by ryanodine receptors (27) and/or inositol 1,4,5-trisphosphate receptors (IP3Rs) (7, 19, 55, 56). Surprisingly, hormone release from EE cells that produce GIP, glucagon-like peptide-1, CCK, or somatostatin, but not chro mogranin A or serotonin, appears to be mostly independent from KATP channels and IP3Rs (42, 61). EE cells that produce secretin or substance P express a heterogeneous phenotype with respect to expression of KATP channels and IP3Rs. Hormone release from GIP/Ins cells is not regulated by ryanodine receptors (unpublished observation). Therefore, different subtypes of EE cells exhibit unexpected complexity, heterogeneity, and novelty concerning the molecules that regulate hormone release.

Bombesin, PMA, protein hydrolysates, glycerolaldehyde, and methylpyruvate are secretagogues for GIP/Ins cells (42). However, it is unknown which signaling pathways are activated by these secretagogues. Protein kinase D (PKD) is a serine/
threonine protein kinase regulated by diacylglycerol signaling (58). Bombesin and phorbol esters stimulate proliferation of 3T3 cells by activating PKD via a PKC-dependent, bisindolylmaleimide I (GF-1)-inhibitable signaling pathway (67, 68). Thus bombesin and PMA could potentially stimulate hormone release from GIP/Ins cells by activating PKC/PKD signaling. In many systems, AMP-activated protein kinase (AMPK) activity is inhibited by nutrients, which coordinately turns on ATP-consuming pathways and inhibits ATP-generating pathways (20, 21, 45). Hydrolyzed proteins, glyceraldehyde, and methylpyruvate are nutritionally rich compounds. Thus these secretagogues could potentially regulate hormone release from GIP/Ins cells by inhibiting AMPK activity. Results presented in this paper indicate that, in GIP/Ins cells, PKC signaling regulates hormone release stimulated by PMA and bombesin but not nutrients. Conversely, nutrients but not PMA or bombesin regulate AMPK signaling, but AMPK-related kinases rather than AMPK itself may regulate nutrient-stimulated hormone release. Furthermore, signaling via bombesin and nutrients is independent and additive, which would allow K cells to independently respond to neural vs. nutritional stimuli.

EXPERIMENTAL PROCEDURES

Cells and culture conditions. GIP/Ins Clone 10 cells are GIP-producing EE cells that were engineered to express the human insulin gene. These cells secrete both insulin and GIP in response to secretagogues (42, 60). Thus insulin release is a surrogate marker for GIP secretion in these cells. Cells were cultured in an atmosphere of 5% CO₂-95% air and 100% humidity in DMEM containing 10% FBS as previously described (42).

Insulin secretion. Insulin secretion was measured essentially as described (47). Briefly, cells were plated at ~10⁵ cells/well in 12-well plates. When ~80% confluent, cells were washed twice with secretion buffer [Kreb's-Ringer bicarbonate buffer containing HEPES plus 0.1% BSA (KRBB-Alb)] (47) and then preincubated at 37°C in secretion buffer. One hour later, buffer was replaced with fresh KRBB-Alb containing the appropriate secretagogue(s). When more than one secretagogue was added to cells, both compounds were added at the same time and not sequentially. Thirty minutes later, assay media were collected, centrifuged to remove any detached cells, and assayed for human insulin by RIA. Bombesin (Calbiochem, San Diego, CA) was added to the cells from a 100× stock solution prepared in KRBB-Alb. Meat hydrolysate (Sigma, St. Louis, MO) was prepared as a 20% (wt/vol) stock solution in PBS. Methylpyruvate, glyceraldehyde (Sigma), and 5-aminomidazole-4-carboxamide-1-β-β-ribofuransoside (AICAR; Calbiochem) were prepared as 100× stock solutions in water. GF-1 (Sigma) was prepared as a 100× stock in DMSO. GF-1 and AICAR were included during the preincubation and also added along with the indicated secretagogue. All values are the average of quadruplicate samples.

Western blot analysis. Cells were treated exactly as described for insulin secretion assays. After collection of the KRBB-Alb, cells were washed twice with ice-cold PBS and lysed directly in 1× SDS-PAGE gel loading buffer. Cell proteins were then separated by SDS-PAGE and transferred to nitrocellulose membranes. Membranes were stained with Ponceau S to confirm that samples were equally loaded and transferred. The names and catalog numbers for antibodies obtained from Cell Signaling Technology (Beverly, MA) are as follows: PKD/PKCα antibody, no. 2052; phospho-PKD/PKCα (Ser⁷⁴⁴/⁷⁴⁸) antibody, no. 2054; phospho-PKD/PKCα (Ser⁹¹⁶) antibody, no. 2051; α-AMPK antibody, no. 2532; and phospho-α-AMPK (Thr²⁷²) antibody, no. 2535. The names and catalog numbers for antibodies obtained from Upstate USA (Charlottesville, VA) are as follows: anti-acetyl-CoA carboxylase, no. 07-439, and anti-phospho-acetyl-CoA carboxylase (Ser⁷⁹), no. 07-303. Antibodies to Kidins220 phosphorylated at Ser⁹¹⁶ (no. K1725-01) were obtained from United States Biological (Swampscott, MA). Western blots were performed according to protocols provided with each antibody.

TRANSIENT TRANFECTION AND HUMAN GROWTH HORMONE SECRETION ASSAYS. These assays are similar to those described in which MIN6 insulinoma cells were cotransfected with a human growth hormone (hGH) reporter construct plus a cDNA that encodes a putative regulatory protein (11, 31). The transiently produced hGH is stored by the endocrine cells and is released along with insulin after stimulation with appropriate secretagogues. Like MIN6, GIP/Ins cells store the transiently produced hGH and release it with appropriate GIP secretagogues. Thus, by transfecting cDNAs that encode hGH along with cDNAs that encode constitutively active or dominant negative AMPKs or PKDs, we can determine the effects of these protein kinases on secretagogue-stimulated hormone release from GIP/Ins cells. GIP/Ins cells were plated at 10⁴ cells/well in 12-well dishes. The next day, quadruplicate wells of cells were transfected with the appropriate cDNAs (see below). Using the LT-1 transfection reagent (Panvera, Madison, WI) according to the manufacturer’s protocol. The following day, cells were washed twice with fresh media, refed, incubated for an additional 24 h, and then assayed for hGH release using the insulin secretion assay protocol. hGH levels were determined with a high-sensitivity ELISA (Abazyme, Needham, MA). Cells in each well were transfected with 0.25 μg of the pTKGH hGH reporter construct (Nichols Diagnostic, San Clemente, CA) plus 0.25 μg of the indicated control or test plasmid(s). The constitutively active α₁-AMPK, dominant negative α₁-AMPK, and dominant negative α₂-AMPK constructs in pCDNA3 were generously provided by Dr. David Carling (Imperial College School of Medicine, University of London, London, UK; see Refs. 52, 63). Constitutively active (ΔPH) and kinase dead (K612W; dominant negative) PKD1 constructs in pEGFP-N1 were generously provided by Dr. Angelika Hausser (Institute for Cell Biology and Immunology, University of Stuttgart, Stuttgart, Germany; see Refs. 23, 28).

Isolation of pools of stably transfected GIP/Ins cells. GIP/Ins cells were plated in 100-mm dishes. The next day, cells were transfected with 1 μg of pRES/hygro (BD Biosciences Clontech, Palo Alto, CA) plus 9 μg of pCDNA3, constitutively active AMPK, or dominant negative α₁- plus α₂-AMPKs (4.5 μg of each). Forty-eight hours later, cells were refed complete media containing hygromycin (400 μg/ml). The next day, cells were trypsinized and replated at different densities. Cells plated at low densities were allowed to grow as individual colonies, which allowed estimation of the number and size of clones from cells transfected with each set of plasmids. Cells replated at higher dilutions were trypsinized and maintained as pools until selection was complete (4 wk). Hygromycin treatment killed 100% of nontransfected cells with the use of this protocol.

RESULTS

PMA and bombesin but not nutrients activate PKC signaling in GIP/Ins cells. Bombesin and bombesin-like peptides stimulate hormone release from gut endocrine cells in the absence of additional agonists (29, 32, 38). In many cell systems, bombesin and PMA activate PKD signaling via a PKC-dependent, GF-1-inhibitable signaling pathway. Phosphorylation of PKD at Ser⁷⁴⁴/⁷⁴⁸ or Ser⁹¹⁶ profoundly increases PKD activity (26, 67, 68). Therefore, we determined whether secretagogues increased phosphorylation of these residues in GIP/Ins cells. Cells were preincubated for 60 min in the absence of secretagogues, treated for the indicated time with secretagogues, and then analyzed by Western blots with the use of antibodies against total or specifically phosphorylated forms of PKD. As shown in Fig. 1, bombesin profoundly increased phosphorylation of PKD on Ser⁷⁴⁴/⁷⁴⁸. Maximal stimulation was observed.
within 15 min, and increased phosphorylation was maintained for at least 90 min (Fig. 1). Throughout this period, total PKD levels or PKD phosphorylated at Ser916 remained relatively constant. Addition of PMA to GIP/Ins cells resulted in similar patterns of PKD expression and phosphorylation in a dose-dependent fashion (not shown). Meat hydrolysate is a nutrient-rich mixture of peptides and amino acids that would be present in the lumen of the gut after ingestion of a meal. Stimulation of GIP/Ins cells with meat hydrolysate did not alter total PKD levels or PKD phosphorylation at Ser744/748 or Ser916. GIP/Ins cells do not secrete hormones in response to glucose (42, 60). However, glyceraldehyde and methylpyruvate are nutrients that bypass glycolysis and stimulate hormone release from these cells (42, 25, 28). Conversely, transient overexpression of a K612W kinase dead PKD (PKD-KD) has been shown to completely inhibit bombesin-stimulated PKD phosphorylation at Ser744/748 but had no effect on phosphorylation at Ser916. GF-1 had no apparent effect on PKD phosphorylation in cells treated with meat hydrolysate. GF-1 completely prevented bombesin- and PMA-stimulated hormone release but had no affect on hormone release from cells treated with meat hydrolysate (Fig. 3 and not shown). Therefore, PKC increases PKD phosphorylation and also stimulates bombesin- or PMA- but not nutrient-stimulated hormone release.

Kidins220 is selectively expressed in the brain and in neuroendocrine cells and is phosphorylated on Ser919 by activated PKD (24). Thus Western blots were probed with antibodies specific for Kidins220 phosphorylated on Ser919. Compared with unstimulated cells at every time point examined, addition of bombesin, meat hydrolysate, glyceraldehyde, or methylpyruvate did not alter phosphorylation of Kidins220 at Ser919 (Fig. 1 and not shown), suggesting that although PKD phosphorylation is increased, PKD activity may not regulate hormone release from GIP/Ins cells. To directly test this hypothesis, a transient hGH secretion assay was utilized to determine whether constitutively active and dominant negative (kinase dead) PKDs (in pEGPF-N1) perturb secretagogue-stimulated hormone release from GIP/Ins cells (see EXPERIMENTAL PROCEDURES). Deletion of the pleckstrin homology domain of PKD (PKD-ΔPH) has been shown to produce a constitutively active form of PKD (23, 25, 28). Deletion of the pleckstrin homology domain of PKD (PKD-ΔPH) has been shown to produce a constitutively active form of PKD (23, 25, 28). Deletion of the pleckstrin homology domain of PKD (PKD-ΔPH) has been shown to produce a constitutively active form of PKD (23, 25, 28). Deletion of the pleckstrin homology domain of PKD (PKD-ΔPH) has been shown to produce a constitutively active form of PKD (23, 25, 28).
to suppress PKD-mediated NF-κB-dependent gene expression (28). Empty pEGFP-N1 and pCDNA3 vectors served as controls. As shown in Fig. 4, GIP/Ins cells transiently expressing the hGH plus control vectors secrete hGH. As noted for insulin release from nontransfected cells (Fig. 3), hGH secretion was increased after addition of bombesin. Importantly, the kinase dead PKD did not suppress bombesin-stimulated hGH release, and the constitutively active PKD did not increase basal hGH release to levels observed after addition of bombesin. Thus PKD activity does not regulate hormone release from GIP/Ins cells. However, because basal hGH release was similarly increased in cells expressing either PKD construct, it is possible that the transiently expressed PKDs can interact with and sequester proteins that inhibit hormone release from GIP/Ins cells. Taken together, these results indicate that bombesin- and PMA-stimulated hormone release from GIP/Ins cells is PKC dependent and does not require PKD activity.

**Fig. 3.** Inhibition of PKC prevents bombesin- but not meat hydrolysate-stimulated hormone release from GIP/Ins cells. Cells were treated with or without GF-1 plus bombesin (A) or meat hydrolysate (B) as described in Figs. 1 and 2. Secretion buffer was collected after 60 min and assayed for human insulin. Note that GF-1 inhibits bombesin-stimulated insulin release and has no effect on meat hydrolysate-stimulated insulin release. GF-1 also completely inhibited PMA-stimulated hormone release (not shown).

Addition of meat hydrolysate caused a rapid and profound decrease in AMPK phosphorylation that was maintained for at least 90 min. Glyceraldehyde and methylpyruvate also inhibited phosphorylation of AMPK at Thr172 (Fig. 2). Conversely, treatment of GIP/Ins cells with bombesin (Figs. 1 and 2) or PMA (not shown) did not reduce AMPK phosphorylation.

Activated AMPK phosphorylates acetyl-CoA carboxylase (ACC) at Ser79 (2, 11). As shown in Fig. 1, addition of meat hydrolysate to GIP/Ins cells caused a rapid and profound decrease in phosphorylation of ACC at Ser79 that paralleled the decrease in AMPK phosphorylation at Thr172. Similar decreases were noted in AMPK and ACC phosphorylation when GIP/Ins cells were treated with glyceraldehyde or methylpyruvate (Fig. 2). Conversely, treatment of cells with bombesin (Figs. 1 and 2) or PMA (not shown) had no affect on the phosphorylation states of AMPK or ACC. Therefore, nutritional secretagogues but not bombesin or PMA rapidly inhibit AMPK activity and signaling in GIP/Ins cells.

**Fig. 4.** PKD and AMPK do not regulate hormone release from GIP/Ins cells. GIP/Ins cells were transiently cotransfected with a human growth hormone (hGH) reporter construct plus the indicated control, PKD, or AMPK expression vector(s). Cells were then treated with no secretagogue or with bombesin or meat hydrolysate and assayed for hGH released into the medium. pCDNA3, pCDNA3 vector with no insert; EGFP, pEGFP-N1 vector with no insert; PKD-ΔPH, constitutively active PKD in pEGFP-N1; PKD-KD, kinase dead PKD in pEGFP-N1; CA-AMPK, constitutively active AMPK in pCDNA3; DN-AMPK, equal amounts of dominant negative α₃- plus α₃-AMPKs in pCDNA3. Each condition was tested in quadruplicate, and results are means ± SE. Similar results were obtained in a second independent experiment. The pCDNA3 and pEGFP controls for A and B are the same samples. Note that cells expressing the constitutively active vs. kinase dead or dominant negative constructs respond similarly to all test conditions, indicating that PKD or AMPK activity does not regulate hormone release from GIP/Ins cells.

**Nutrients but not bombesin or PMA inhibit AMPK activity in GIP/Ins cells.** Next, we investigated whether nutrients regulate AMPK rather than PKC activity in GIP/Ins cells. AMPK activity is profoundly increased by phosphorylation at Thr172 (20, 45). Typically, AMPK phosphorylation and activity are high under nutrient-poor conditions. GIP/Ins cells were treated as described above and then analyzed for total AMPK or AMPK phosphorylated at Thr172. In the absence of secretagogues, AMPK is highly phosphorylated at Thr172 (Fig. 1).
AMPK does not regulate nutrient-stimulated hormone release from GIP/Ins cells. AICAR is a cell-permeable analog of adenosine that can activate AMPK in vivo (53). AICAR (0.1–3 mM) did not inhibit meat hydrolysate-stimulated hormone release from GIP/Ins cells (not shown). However, addition of 1 mM AICAR reversed much of the meat hydrolysate-stimulated decrease in AMPK phosphorylation (Fig. 2) but did not prevent the meat hydrolysate-regulated decrease in ACC phosphorylation (Fig. 2). These observations suggest that AMPK may not be the major regulator of ACC phosphorylation. Two sets of experiments were conducted to directly determine whether AMPK regulates nutrient-stimulated hormone release from GIP/Ins cells. First, cells were transiently transfected with the hGH reporter construct along with cDNA vectors that encode a constitutively active AMPK vs. dominant negative α1- plus α2-AMPKs, as described for PKD. Both dominant negative forms of AMPK were included, since inhibition of one AMPK isoform can increase expression of the other (36). Control cells were transfected with empty pCDNA3 or enhanced green fluorescent protein (EGFP) vectors. Similar to insulin release from nontransfected cells, GIP/Ins cells transfected with the hGH reporter plus either control vector secreted increased amounts of hGH in response to bombesin and meat hydrolysate (Fig. 4B). However, the response to meat hydrolysate was somewhat blunted compared with that of nontransfected cells (Fig. 3). Basal hGH release was increased to a similar extent in cells transfected with the dominant negative or constitutively active AMPKs. Furthermore, expression of the constitutively active AMPK failed to inhibit meat hydrolysate-stimulated hGH release. As expected, expression of the AMPK plasmids had no effect on bombesin-stimulated hGH release. Next, separate dishes of GIP/Ins cells were transfected with an empty pCDNA vector or with constitutively active or α1- plus α2-dominant negative AMPK plasmids. Pools of stably transfected cells were then isolated and assayed for insulin release as described in Fig. 3. Each pool was composed of ~1,500 similarly sized clones, suggesting that AMPK activity did not alter cell growth or viability (not shown). Addition of meat hydrolysate or glyceraldehyde increased insulin release from each of the three different pools of transfected cells threefold and fourfold, respectively (not shown). Therefore, nutrients but not PMA or bombesin inhibit phosphorylation of AMPK in GIP/Ins cells. However, AMPK does not regulate hormone release.

Bombesin- and nutrient-signaling pathways independently and additively regulate hormone release from GIP/Ins cells. The previous results indicate that bombesin and nutrients regulate different signaling pathways. Nutrient- and bombesin-signaling pathways could converge at a common downstream point or else represent parallel independent signaling pathways. To distinguish between these two possibilities, GIP/Ins cells were treated with varying concentrations of bombesin and/or meat hydrolysate. As shown in Fig. 5, hormone release was maximally stimulated by 10^{-5} M bombesin alone (3-fold) or 0.3% meat hydrolysate alone (2.5-fold). Addition of 10^{-5} M bombesin plus 0.3% meat hydrolysate resulted in a sixfold increase in hormone release. Therefore, bombesin- and nutrient-regulated signaling pathways are working in an independent and additive manner to stimulate hormone release from GIP/Ins cells.

**Discussion**

Hormones produced by EE cells regulate a variety of important gastrointestinal and whole animal physiological events. Therefore, it is critical for EE cells to be able to tightly control production and release of specific EE cell products. To accomplish this, each specific subtype of EE cell must be able to sense and respond not only to changes in specific luminal contents but also to neurotransmitters released by adjacent neurons as well as to other regulatory molecules produced by additional intestinal and nonintestinal cell types (35). Previous studies from this laboratory have shown that different subtypes of EE cells utilize remarkably distinct and heterogeneous sets of proteins to regulate hormone release (42, 60, 61).

In many cells, bombesin and PMA activate PKD signaling in a PKC-dependent, GF-1-inhibitable manner (9, 34, 64, 67, 68). Bombesin and PMA but not nutrients increased phosphorylation of PKD on Ser744/748 but not on Ser916. Because GF-1 inhibited bombesin and PMA stimulated phosphorylation of PKD at Ser744/748, these two secretagogues activate PKC in GIP/Ins cells. Site-directed mutagenesis of positions 744/748 of PKD has demonstrated that phosphorylation of these two residues alone is sufficient to regulate PKD activity (26, 65, 66). However, addition of bombesin or PMA did not increase phosphorylation of Kidins220, the only known in vivo substrate for activated PKD, and overexpression of constitutively active or dominant negative PKDs did not perturb secretagogue-stimulated hormone release. Because GF-1 inhibited phosphorylation of PKD and also prevented bombesin- and PMA-stimulated hormone release, the effects of bombesin and PMA are mediated by PKC and are mostly independent of PKD activity. There have been two other reports that examined the potential role for PKD in regulating hormone release from gut endocrine cells. In agreement with our results, Moore et al. (38) presented evidence that PKC but not PKD regulates hormone release from purified human antral gastrin-producing EE cells. The authors also showed that gastrin-producing EE cells express at least six different isoforms of PKC. Thus it will be important to determine which PKC isoforms are expressed by GIP/Ins cells and to evaluate the physiological role for each isoform. Conversely, Li et al. (32) concluded that PKD plays...
a central role in regulating neurotensin release from BON cells. However, in these latter studies, expression of a constitutively active PKD in BON cells increased basal neurotensin release only \( \sim 2 \)-fold, whereas PMA increased neurotensin release \( \sim 10 \)-fold. In addition, BON cells transfected with the gastrin-releasing peptide receptor exhibit a 10-fold increase in neurotensin release after addition of bombesin. However, after transfection with PKD small interfering RNA, these same cells exhibited only a twofold reduction in bombesin-stimulated neurotensin release even though PKD expression and activation appeared to be completely blocked. Thus PKC may also be much more important than PKD for regulating neurotensin release.

Phosphorylation of AMPK at Thr\(^ {172} \) greatly increases its enzyme activity (20). Addition of meat hydrolysate, glyceroldehyde, or methylpyruvate, but not PMA or bombesin, to GIP/Ins cells resulted in decreased phosphorylation of AMPK at Thr\(^ {172} \). AMPK signaling also appeared to be inhibited, since these same nutrients suppressed phosphorylation of ACC. However, forced expression of constitutively active vs. dominant negative AMPKs did not perturb secretagogue-stimulated hormone release from GIP/Ins cells. These results suggest that AMPK signaling does not regulate hormone release but may be important for regulating other aspects of K cell physiology. In contrast to our results, forced expression of a dominant negative AMPK in MIN6 insulinoma cells led to unregulated insulin release, whereas forced expression of a constitutively active AMPK blocked glucose-stimulated insulin release (11). These results further illustrate the remarkable differences in the proteins that regulate hormone release from islet \( \beta \)-cells vs. GIP-producing EE cells.

It was initially unclear why AICAR, which can activate AMPK in other systems, failed to inhibit insulin release from GIP/Ins cells. One possible explanation is that GIP/Ins cells do not efficiently metabolize AICAR. Alternatively, there is some evidence that, in islet \( \beta \)-cells, AICAR can be phosphorylated to ZMP, which can then be phosphorylated further to ZTP, an ATP mimic. This, in turn, could stimulate depolarization and subsequent hormone release from \( \beta \)-cells by inhibiting \( K_{\text{ATP}} \) channel activity without inhibiting AMPK activity (45). However, \( K_{\text{ATP}} \) channels do not regulate hormone release from GIP/Ins cells (42, 60). Therefore, it is possible that additional intermediates derived from metabolism of AICAR could inhibit rather than activate AMPK. A much more intriguing and unifying explanation for our results is that nutrients control hormone release from GIP/Ins cells by regulating activity of AMPK-related kinases, rather than AMPK itself. Twelve human AMPK-related kinases have recently been identified and partially characterized (33). Like AMPK, 11 of these AMPK-related kinases are phosphorylated in their T-loops by LKB1, an AMPK kinase. Phosphorylation at these sites increased enzyme activity \( \geq 50 \)-fold. In contrast to AMPK, the activity of LKB1 or the AMPK-related kinases is not increased by AICAR. Although there were marked differences in the relative rates, all of the AMPK-related kinases also phosphorylated each of the three peptides that are substrates for AMPK. Therefore, our results suggest that AMPK-related kinases regulate hormone release from GIP/Ins cells, since 1) AICAR activated AMPK but did not inhibit phosphorylation of ACC, a substrate for AMPK; 2) AICAR did not inhibit nutrient-stimulated hormone release; and 3) forced expression of AMPKs did not perturb hormone release from GIP/Ins cells.

It was predicted that PMA and bombesin vs. nutrients would control hormone release by regulating different signaling pathways. However, it was unexpected to see that there was no apparent cross talk between the PKC and AMPK/AMPK-related kinase signaling pathways and that hormone release regulated by these two pathways was additive. Because bombesin-like peptides are produced by enteric neurons, these results imply that K cells independently sense and respond to nutritional and neuronal stimuli.

As already discussed, K cells do not utilize many of the same regulatory proteins to control hormone release that are used by other endocrine, excitatory, and some EE cells (42, 60, 61). However, evidence presented in this paper indicates that GIP secretagogues regulate two conserved signaling pathways in GIP/Ins cells. This implies that steps leading to the activation of PKC and/or inactivation of AMPK/AMPK-related kinases may represent critical steps in regulating K cell physiology and GIP release in vivo. This also implies that PKC and AMPK/AMPK-related kinase signaling can regulate hormone release from other subtypes of EE cells and that cell-specific differences in the regulatory cascades that activate or inactivate these kinases control release of other hormones. For example, cell-specific molecules that regulate bombesin receptor signaling could potentially confer differential responses to bombesin-like peptides in distinct subtypes of EE cells. With respect to nutrients, GIP is released in response to a set of amino acids different from those that stimulate gastrin and CCK release (59). Therefore, amino acid transporters present on one type of EE cell but not on another could potentially initiate cell-specific cascades that inhibit AMPK/AMPK-related kinase signaling and thus stimulate release of hormones from only a specific type of EE cell. However, it is also possible that different subtypes of EE cells express distinct AMPK-related kinases, and each of these kinases can be regulated by metabolites derived from different nutrients.

There is a large body of biochemical, physiological, and genetic evidence indicating that GIP promotes obesity and insulin resistance (4, 6, 8, 10, 14–17, 22, 30, 37, 39, 41, 46, 51, 62). Furthermore, mice lacking GIP receptors do not exhibit any serious detrimental phenotypes (37). Therefore, inhibition of GIP receptor signaling is a potential strategy to ameliorate obesity and insulin resistance. This could be accomplished by development of GIP receptor antagonists. An alternative and complementary approach would be to inhibit GIP release from K cells. One advantage of this latter approach is that potential drugs could be delivered orally to K cells, which could minimize adverse side effects due to systemic drug delivery. A second advantage is that different subtypes of EE cells utilize distinct proteins to control hormone release (42), suggesting that drugs that inhibit GIP release, but do not adversely affect other cell populations, could be rationally developed (60, 61). Therefore, as a first step in developing potential anti-obesity drugs, it is critical to elucidate the mechanisms that regulate GIP release from K cells so that K cell-specific components of these pathways can be defined. Studies are underway to determine the roles of PKC, PKD, AMPK, and AMPK-related kinase signaling pathways in regulating hormone release from K cells in vivo, as well as from other subtypes of EE cells, and to identify cell-specific components of these pathways.
ACKNOWLEDGMENTS

We thank Drs. David Kipnis and Ernesto Bernal for helpful discussions.

GRANTS

This work was supported in part by a Career Development Award from the American Diabetes Association (B. M. Wice) and the Washington University Diabetes and Research Training Center (National Institute of Diabetes and Digestive and Kidney Diseases Grant 5-P60-DK-20579).

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