Studies with GIP/Ins cells indicate secretion by gut K cells is \( K\text{ATP} \) channel independent

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ENTEROENDOCRINE (EE) CELLS are a complex population of diffusely distributed hormone-producing intestinal epithelial cells that play important roles in regulating and integrating many aspects of gastrointestinal and whole animal physiology (1, 32, 48, 52, 57, 66). Although they represent <1% of the intestinal epithelial cells, EE cells as a whole represent the largest endocrine organ in the body. There are at least 16 different subpopulations of EE cells based on the major product(s) produced and secreted by individual cells (1, 48, 57). The specific product(s) is dependent on a cell’s position along the crypt to villus and stomach to colonic axes of the gut (49, 57).

Glucose-dependent insulintropic polypeptide (GIP) and glucagon-like peptide-1 (GLP-1) are EE cell-derived “incretin” hormones that are released in the circulation immediately after ingestion of a meal and potentiate insulin secretion after binding to receptors on islet \( \beta \)-cells (32). GIP is produced and secreted by gut K cells located in the stomach and proximal small intestine, whereas GLP-1 is a product of gut L cells located in the distal small intestine (13, 52, 65, 66, 73). Mice lacking GIP (34) or GLP-1 (53) receptors exhibit impaired first-phase glucose-stimulated insulin release or glucose intolerance, respectively. In addition to effects on glucose homeostasis, GIP promotes obesity in mice fed a high-fat diet (33). Thus it is important to understand the molecular mechanisms that regulate GIP and GLP-1 secretion.

Incretin-producing EE cells respond to changes in the concentrations of luminal nutrients but are refractive to changes in the levels of nutrients in the blood (11, 13, 52). Glucose (5, 62, 65), protein hydrolysates (70), specific amino acids (64), and fat (12) are the major nutrients that stimulate GIP release. Gastrin-releasing peptide (GRP), a hormone produced and secreted by enteric neurons, also stimulates GIP release (46). Conversely, somatostatin (SST) inhibits secretion from gut K cells (32, 66). GLP-1 secretion is also under nutritional (11, 44), hormonal (4, 45), and neuronal (47) control (10, 18). Interestingly, GIP stimulates GLP-1 secretion from gut L cells (4, 45).

Because GIP is secreted by gut K cells with a temporal pattern and in response to similar nutrients as insulin secretion by islet \( \beta \)-cells, it has been proposed that engineering gut K cells to produce insulin is a...
potential gene therapy to treat diabetes (7, 39). To begin to test this hypothesis, novel GIP-producing cell lines were established and engineered to express the human insulin gene (GIP/Ins cells). Like K cells in vivo, GIP/Ins cells secreted both insulin and GIP in response to the GIP secretagogues arginine, bombesin, and protein hydrolysates (39). However, glucose failed to stimulate hormone release from the cells even though they express glucokinase, the major glucose-sensing enzyme used by islet β-cells and hepatocytes. This observation is consistent with published results that demonstrated glucose-stimulated GIP release was dependent on sugar uptake by enterocytes (62) and suggests that gut K cells may not directly sense glucose in the lumen of the gut (see Ref. 39 for detailed discussion). In contrast to glucose, glyceraldehyde and methyl pyruvate were good secretagogues for GIP/Ins cells, indicating that these cells can sense changes in the intracellular concentrations of specific metabolic intermediates.

We have begun to study the regulation of gut K cell physiology by using the well-characterized islet β-cell as a model (17, 21). In islet β-cells, glucose metabolism results in an increase in the intracellular ATP-to-ADP ratio. This, in turn, inhibits the ATP-dependent potassium (KATP) channel, resulting in cell depolarization, influx of calcium, and finally exocytosis of insulin from secretory granules. Methyl pyruvate is thought to stimulate insulin release from β-cells by increasing the mitochondrial ATP levels (20, 31). However, the situation concerning glyceraldehyde-stimulated insulin release is more complicated. After phosphorylation to glyceraldehyde-3-phosphate by triose kinase, glyceraldehyde can be metabolized via glycolysis and the tricarboxylic acid cycle to ultimately increase the intracellular ATP-to-ADP ratio. If this pathway is active in K cells, lack of glucose-stimulated insulin secretion by GIP/Ins cells could result from an inability to metabolize glucose in the early steps of glycolysis. However, it has also been proposed that glyceraldehyde, in the presence of Pi, can be directly phosphorylated by glyceraldehyde phosphate dehydrogenase to generate the nonmetabolizable intermediate glyceraldehyde-1-phosphate (24). This reaction would increase the intracellular NADH-to-NAD ratio and, consequently, mitochondrial ATP synthesis. Taken together, these observations raise the possibility that glucose cannot be metabolized to glyceraldehyde-3-phosphate by GIP/Ins cells and methyl pyruvate, and glyceraldehyde-stimulated hormone release by increasing the intracellular ratio of ATP to ADP independently of early steps in glycolysis. However, RT-PCR analyses of mRNAs prepared from 10 independently derived GIP-producing cell lines indicated that these cells express only very low levels of inward rectifying potassium channel 6.1 (Kir 6.1), inward rectifying potassium channel 6.2 (Kir 6.2), sulfonylurea receptor 1 (SUR 1), sulfonylurea receptor 2A (SUR 2A), and sulfonylurea receptor 2B (SUR 2B), suggesting that nutrient-stimulated hormone secretion by gut K cells may be independent of KATP channels altogether (39). This hypothesis is also supported by the observation that sulfonylurea compounds and potassium channel opening (KCO) drugs had relatively little effect on secretion by GIP/Ins cells (39). Interestingly, islet β-cells exhibit both KATP channel-dependent and -independent mechanisms of secretion (50). To begin to sort out biochemical and molecular mechanisms that regulate secretion from K cells, the intracellular concentrations of specific glycolytic and citric acid cycle intermediates were determined in GIP/Ins cells cultured in the absence of glucose vs. 25 mM glucose, glyceraldehyde, or methyl pyruvate. As a control, similar measurements were performed using insulin-secreting MIN6 cells, a well-characterized, glucose-responsive, β-cell line (35, 67). Results of these analyses indicated that glucose is rapidly metabolized by the GIP/Ins cells and secretion is independent of the intracellular ATP-to-ADP ratio. These results are consistent with the RT-PCR data indicating that KATP subunit channels are expressed at very low levels in GIP/Ins cells. Double-label immunohistochemical techniques were then used to demonstrate that gut K cells in vivo, as well as gut endocrine cells that secrete GLP-1, SST, and CCK, do not express either Kir 6.1 or Kir 6.2. Therefore, depolarization by these gut endocrine cells is fundamentally different from that by islet β-cells. However, several specific subpopulations of EE cells do express Kir 6.2, revealing an unexpected level of complexity concerning mechanisms of hormone secretion by different subtypes of EE cells.

MATERIALS AND METHODS

Cells and culture conditions. All cells were cultured in an atmosphere of 5% CO2-95% air and 100% humidity. MIN6 cells (passage 25–40) were cultured in DMEM containing 15% FCS, as previously described (39, 67), and GIP/Ins cells were cultured in DMEM containing 10% FCS (39).

Hormone secretion assays. Insulin or GIP secretion was measured as previously described (39). Briefly, GIP/Ins or MIN6 cells were plated in 12-well tissue culture dishes. When nearly confluent, cells were washed two times with PBS (containing calcium and magnesium) and then preincubated for 60 min at 37°C in insulin secretion assay buffer (glucose-free KRBH-Alb; see Ref. 51). Cells were then refed secretion buffer containing the indicated secretagogue. Later (90 min), buffers were collected, centrifuged to remove detached cells, and then assayed for human insulin or GIP production by RIA.

Measurement of intracellular metabolite levels. Metabolite levels were determined in cells cultured under the same conditions that were used to measure hormone secretion (39) except cells were plated in 60-mm dishes (51). Thirty minutes after addition of assay buffer containing the indicated secretagogue, cells were washed two times with PBS (containing calcium and magnesium) and then preincubated for 60 min at 37°C in insulin secretion assay buffer (glucose-free KRBH-Alb; see Ref. 51). Cells were then refed secretion buffer containing the indicated secretagogue. Later (90 min), buffers were collected, centrifuged to remove detached cells, and then assayed for human insulin or GIP production by RIA.

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olite values were normalized to the amount of protein in the original cell lysate. Alkali-treated extracts were used for the determination of ATP, ADP, pyruvate, fructose 1,6-bisphosphate (F-1,6-P2), and citrate. Glucose 6-phosphate (G-6-P) and glutamate were measured on acid-treated extracts. Metabolites were assayed in a direct, or linked, coupled enzymatic reaction that either oxidized or reduced NAD(P)(H), as described previously (8, 22, 41, 42, 69). Pyridine nucleotides generated in the enzymatic reaction were measured using a Farrand A4 Fluorometer containing a 7–60 excitation filter and 5–57 plus 3–73 emission filters.

Measurement of lactic acid production. GIP/Ins cells were plated in six-well tissue culture dishes and treated as described for insulin secretion assays (39). After the 90-min incubation with or without secretagogues, secretion buffers were collected and centrifuged to remove detached cells. Lactic acid in the buffer was then determined using lactate dehydrogenase (Sigma Chemical, St. Louis, MO). All values are normalized to the amount of cell protein in each dish.

Antibody staining. The entire mouse small intestine was removed en bloc immediately after the animal was killed and then flushed with PBS followed by freshly prepared 4% paraformaldehyde in PBS. The intestine was then cut longitudinally along its entire length. After fixation for a total of 1 h at room temperature, the intestine was stored in 70% alcohol (at least overnight) and then rolled up from the duodenum to distal ileum. The resulting “Swiss roll” (14, 15) was cut in half with a razor blade along the duodenal to ileal axis and then impregnated with 2% agar in 5% phosphate-buffered formalin. The Swiss rolls were then embedded in paraffin, sectioned, and stained as described below. Other tissues were fixed and embedded in paraffin without the use of agar.

Tissue sections were deparaffinized in xylene and rehydrated in graded alcohols followed by water and then PBS. After antigen retrieval using 1 mM EDTA (38), sections were washed with PBS and then blocked using BACKGROUND-SNIPER (Biocare Medical, Walnut Creek, CA). Sections were then incubated for 60 min at room temperature with the appropriate primary antibodies [diluted to the indicated concentration in Da Vinci Green Antibody Diluent (Biocare Medical)]. After three washes with PBS, sections were incubated for 45 min at room temperature with the indicated secondary antibodies diluted in Da Vinci Green Primary Antibody Diluent. Sections were then washed, and nuclei were stained with bis-benzimide and mounted in PBS-glycerol as described (68).

The following antibodies were used to stain the tissue sections reported here: goat anti-Kir 6.1 (catalog no. sc-11225; 2 μg/ml; Santa Cruz Biotechnology, Santa Cruz, CA); goat anti-Kir 6.2 (catalog no. sc-11228; 2 μg/ml; Santa Cruz Biotechnology); rabbit anti-GIP (catalog no. IHC-7154, 1 μg/ml; Peninsula Laboratories, Torrance, CA); rabbit anti-GLP-1 (catalog no. IHC-7123, 1 μg/ml; Peninsula Laboratories); rabbit anti-SST-14 (catalog no. IHC-8001, 1 μg/ml; Peninsula Laboratories); rabbit anti-substance P (SP; IHC-7451, 1 μg/ml; Peninsula Laboratories); rabbit anti-CCK (catalog no. IHC-61014, 1 μg/ml; Peninsula Laboratories); rabbit anti-CGA (catalog no. 20085, 1:1,000 dilution; Diasorin, Stillwater, MN); rabbit anti-secretin and rabbit anti-serotonin were kindly provided by Dr. Jeffrey Gordon of our university and used at 1 μg/ml. All fluorochrome-conjugated secondary antibodies (Jackson ImmunoResearch, West Grove, PA) were raised in donkeys and pepsin-digested to reduce cross-reactivity with IgG from other species. Secondary antibodies were used at a 1:1,000 dilution.

Quantifying Kir 6.2 and gut hormone double positive cells. Single sections of paraffin-embedded mouse small intestine were incubated with goat anti-Kir 6.2 plus rabbit anti-GIP, GLP-1, secretin, serotonin, SST-14, SP, CCK, or chromogranin A (CGA; see above). Bound primary antibodies were detected using Cy3-conjugated donkey anti-sheep IgG (red) plus FITC-conjugated donkey anti-rabbit IgG (green) secondary antibodies. Individual cells expressing a specific gut hormone (green) were identified using a fluorescence microscope. The filters were then changed to determine whether Kir 6.2 (red) was expressed by that same cell. Typically, a total of 300 green cells was analyzed for coexpression of Kir 6.2.

RESULTS

Glycolysis is not impaired in GIP/Ins cells. Methyl pyruvate (data not shown) and glyceraldehyde, but not glucose, stimulate hormone release from GIP/Ins cells (Fig. 1 and also see Ref. 39). Glyceraldehyde enters glycolysis downstream of phosphofructokinase 1. This raised the possibility that GIP/Ins cells cannot metabolize glucose through early steps of glycolysis. To address this issue, the intracellular concentrations of several glycolytic intermediates were determined in cells cultured under the same conditions used for the hormone secretion assays. Cells were incubated for 1 h in insulin secretion assay buffer (no glucose) to deplete intracellular metabolites and then refed buffer with or without 25 mM glucose. Later (30 min), cells were harvested, extracted, and then assayed for the indicated metabolite. As a control, similar measurements were performed using glucose-responsive MIN6 insuli-
nomina cells. As shown in Tables 1 and 2, addition of glucose caused a 56-fold increase in G-6-P levels in GIP/Ins cells, whereas MIN6 cells exhibited a 7-fold increase in the level of this metabolite. That G-6-P levels increased eightfold more in GIP/Ins cells compared with MIN6 cells indicated that glucose phosphorylation was not limiting and suggested that phosphofructokinase 1, the next regulated step in glycolysis, could possibly be inactive in GIP/Ins cells. However, addition of glucose caused 143- and 38-fold increases in F-1,6-P$_2$ levels in GIP/Ins and MIN6 cells, respectively. Thus phosphofructokinase 1 activity is not limiting in GIP/Ins cells. Finally, the levels of pyruvate, the end product of glycolysis, were determined. Addition of glucose resulted in 2.4- and 2-fold increases in pyruvate levels in GIP/Ins and MIN6 cells, respectively. Therefore, glucose-stimulated increases in the levels of glycolytic intermediates were greater in GIP/Ins vs. MIN6 cells even though glucose stimulates hormone release from MIN6, but not GIP/Ins, cells.

Although the preceding results strongly suggest that glycolysis is not impaired in GIP/Ins cells, it is possible that metabolite levels do not reflect the actual flux of carbon through glycolysis. To address this issue, GIP/Ins cells were incubated for 90 min in insulin secretion assay buffer either with or without 25 mM glucose. The amount of lactic acid in the secretion buffer was then determined. As shown in Fig. 2, GIP/Ins clones 10 and 12 incubated with glucose produced 45 and 75 μg lactate·mg protein$^{-1}$·90 min$^{-1}$, respectively. Next, lactate secretion was measured in GIP/Ins cells treated with glyceraldehyde. It is important to note that glyceraldehyde can be metabolized to lactate via glycolysis or to glyceraldehyde 1-phosphate plus NADH by glyceraldehyde phosphate dehydrogenase. If this latter reaction is operative, the increased NADH levels would drive production of lactate from pyruvate. Thus lactate production from glyceraldehyde-treated cells represents the maximal flux through the downstream reactions of glycolysis. As shown in Fig. 2, metabolism of glyceraldehyde or glucose generated similar amounts of lactate, indicating that flux through glycolysis is not preventing glucose-stimulated hormone release from GIP/Ins cells.

**Secretion by GIP/Ins cells is independent of the intracellular ATP-to-ADP ratio.** The previous results indicate that lack of glycolysis cannot account for the inability of glucose to stimulate hormone release from GIP/Ins cells. Pyruvate generated via glycolysis appears to enter the tricarboxylic acid cycle, since the intracellular concentration of citrate and the glucose-stimulated increase in citrate levels are similar in GIP/Ins and MIN6 cells (Tables 1 and 2). Because the ATP-to-ADP ratio plays a critical role in regulating K$_{ATP}$ channel activity and cell depolarization in islet β-cells, this ratio was determined on GIP/Ins and MIN6 cells treated with glucose, glyceraldehyde, or methyl pyruvate. The addition of glucose or methyl

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**Table 1. Intracellular metabolite levels in GIP/Ins clone 10 and MIN6 cells stimulated for 30 min with 25 mM glucose, glyceraldehyde, or methyl pyruvate.**

<table>
<thead>
<tr>
<th>Nutrient</th>
<th>G-6-P (6)</th>
<th>F-1,6-P$_2$ (3)</th>
<th>Pyruvate (6)</th>
<th>Citrate (3)</th>
<th>Glutamate (3)</th>
<th>ATP (9)</th>
<th>ADP (9)</th>
<th>ATP/ADP</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>GIP/Ins</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>0.05 ± 0.003</td>
<td>0.0027 ± 0.002</td>
<td>1.37 ± 0.20</td>
<td>5.63 ± 0.61</td>
<td>31.3 ± 2.26</td>
<td>6.50 ± 0.45</td>
<td>4.81</td>
<td></td>
</tr>
<tr>
<td>Glucose</td>
<td>2.94 ± 0.30</td>
<td>0.38 ± 0.006</td>
<td>3.24 ± 0.42</td>
<td>5.23 ± 0.61</td>
<td>58.9 ± 2.68†</td>
<td>38.1 ± 3.85</td>
<td>5.69 ± 0.49†</td>
<td>6.69</td>
</tr>
<tr>
<td>Glyceraldehyde</td>
<td>ND</td>
<td>ND</td>
<td>5.41 ± 0.22</td>
<td>ND</td>
<td>33.8 ± 0.73†</td>
<td>25.0 ± 0.40†</td>
<td>9.11 ± 0.29†</td>
<td>2.75</td>
</tr>
<tr>
<td>Me-Pyr</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>9.32 ± 0.95*</td>
<td>41.1 ± 1.16*</td>
<td>5.34 ± 0.39*</td>
<td>7.70</td>
</tr>
<tr>
<td><strong>MIN6</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>0.023 ± 0.002</td>
<td>0.0046 ± 0.002</td>
<td>1.75 ± 0.16</td>
<td>3.48 ± 0.34</td>
<td>41.1 ± 2.65</td>
<td>28.9 ± 1.08</td>
<td>13.3 ± 0.06</td>
<td>2.17</td>
</tr>
<tr>
<td>Glucose</td>
<td>0.156 ± 0.01</td>
<td>0.18 ± 0.007</td>
<td>3.61 ± 0.33*</td>
<td>4.48 ± 0.49†</td>
<td>51.7 ± 2.09†</td>
<td>32.5 ± 1.57</td>
<td>8.23 ± 0.49†</td>
<td>3.94</td>
</tr>
<tr>
<td>Glyceraldehyde</td>
<td>ND</td>
<td>ND</td>
<td>6.45 ± 0.27</td>
<td>ND</td>
<td>34.2 ± 1.45</td>
<td>30.9 ± 2.27†</td>
<td>12.1 ± 0.61†</td>
<td>2.56</td>
</tr>
<tr>
<td>Me-Pyr</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>21.9 ± 1.47†</td>
<td>38.4 ± 0.06†</td>
<td>6.51 ± 0.27†</td>
<td>5.89</td>
</tr>
</tbody>
</table>

**GIP/Ins clone 10** or MIN6 cells were preincubated for 60 min in KRBH-Alb in the absence of glucose to deplete metabolites before 25 mM glucose, glyceraldehyde, or methyl pyruvate (Me-Pyr) was added to parallel cultures. Later (30 min), cells were extracted for measurement of the indicated metabolite. All values are nmol/mg total cellular protein. Nos. in parentheses indicate no. of dishes analyzed for each condition except 1 represents n = 3. †P < 0.005 and *P < 0.05 when measured concentration of a specific metabolite is compared in treated vs. untreated cells. ND, not determined.

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**Table 2. Degree of increase in intracellular metabolites after addition of the indicated nutrient.**

<table>
<thead>
<tr>
<th>Cells</th>
<th>Nutrient</th>
<th>G-6-P</th>
<th>F-1,6-P$_2$</th>
<th>Pyruvate</th>
<th>Citrate</th>
<th>Glutamate</th>
<th>ATP</th>
<th>ADP</th>
<th>ATP/ADP</th>
</tr>
</thead>
<tbody>
<tr>
<td>GIP/Ins</td>
<td>Glucose</td>
<td>56</td>
<td>143</td>
<td>2.4</td>
<td>2.0</td>
<td>1.3</td>
<td>1.2</td>
<td>0.9</td>
<td>1.4</td>
</tr>
<tr>
<td></td>
<td>Glyceraldehyde</td>
<td>3.9</td>
<td></td>
<td>0.8</td>
<td>0.8</td>
<td>1.4</td>
<td>1.4</td>
<td>0.6</td>
<td>1.4</td>
</tr>
<tr>
<td></td>
<td>Me-Pyr</td>
<td></td>
<td></td>
<td>0.2</td>
<td>1.3</td>
<td>0.8</td>
<td>1.6</td>
<td>1.6</td>
<td>1.6</td>
</tr>
<tr>
<td>MIN6</td>
<td>Glucose</td>
<td>6.8</td>
<td>38</td>
<td>2.1</td>
<td>1.5</td>
<td>1.3</td>
<td>1.1</td>
<td>0.6</td>
<td>1.8</td>
</tr>
<tr>
<td></td>
<td>Glyceraldehyde</td>
<td>3.7</td>
<td></td>
<td>0.8</td>
<td>1.1</td>
<td>0.9</td>
<td>1.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Me-Pyr</td>
<td>0.5</td>
<td></td>
<td>1.3</td>
<td>1.5</td>
<td>0.2</td>
<td>2.7</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Values represent the concentration of each metabolite in cells incubated with the indicated nutrient relative to that in cells maintained without glucose. Concentrations are taken from Table 1.
pyruvate to GIP/Ins cells resulted in similar increases in the ATP-to-ADP ratio (1.4- vs. 1.6-fold increases, respectively), suggesting that secretion was independent of ATP-to-ADP ratios. Surprisingly, glyceraldehyde caused a dramatic decrease in the intracellular ATP-to-ADP ratio (just the opposite of what would be expected if this fuel secretagog stimulated secretion via K\textsubscript{ATP} channels). In MIN6 cells, methyl pyruvate caused a greater increase in the ATP-to-ADP ratio than did glucose (2.6- vs. 1.5-fold), yet glucose was a better secretagogue than methyl pyruvate (Fig. 3). These observations suggest that methyl pyruvate stimulated secretion from \(\beta\)-cells by K\textsubscript{ATP}-dependent and -independent mechanisms. However, glyceraldehyde had little effect on the ATP-to-ADP ratio in MIN6 cells, indicating that, as was observed with GIP/Ins cells, glyceraldehyde stimulates hormone secretion independently from effects on the intracellular ATP-to-ADP ratio. Interestingly, addition of glyceraldehyde to MIN6 or GIP/Ins cells caused a nearly fourfold increase in the intracellular levels of pyruvate, whereas glucose increased pyruvate levels only approximately twofold for both cell types.

\textit{Gut K cells in vivo do not express Kir 6.1 or Kir 6.2.}\n
The preceding results suggest that secretion by gut K cells is K\textsubscript{ATP} channel independent. This result would be consistent with our published observation that GIP-producing cell lines express very low levels of Kir 6.1, Kir 6.2, SUR 1, SUR 2A, and SUR 2B. To address the issue of whether the GIP/Ins cells reflect what occurs in vivo, double-label immunohistochemical studies were performed to determine whether gut K cells express K\textsubscript{ATP} channels. These channels are expressed in a variety of cell types, including islet \(\beta\)-, vascular smooth muscle, and heart cells, and are composed of four subunits of a regulatory protein SUR and four subunits of a Kir isoform (74). Kir 6.2 is the major Kir subunit expressed in islet \(\beta\)-cells. Thus sections of paraffin-embedded mouse pancreas were stained with goat anti-Kir 6.2 plus guinea pig anti-insulin antibodies (Fig. 4). As expected, Kir 6.2 was expressed in insulin-containing islet \(\beta\)-cells. Next, sections of paraffin-embedded mouse small intestine were stained with goat anti-Kir 6.2 plus rabbit anti-GIP antibodies. As shown in Fig. 5, the Kir 6.2 antibodies recognized an antigen present in a rare population of cells scattered throughout the intestinal epithelium (red). Preincubation of this antibody with the peptide against which it was raised abolished all staining (data not shown). This pattern of staining is consistent with Kir 6.2 being expressed in EE cells. However, when the same section was examined for expression of GIP (green), it was apparent that Kir 6.2 and GIP expression was discordant. A quantitative analysis revealed that only 2.6% of the gut K cells expressed Kir 6.2 (Table 3). Next, sections of mouse small intestine were examined for expression of Kir 6.1 plus GIP. As shown in Fig. 6, only very weak, diffuse cytoplasmic staining of the intestinal epithelium was observed with the antibodies directed against Kir 6.1. Importantly, the antibodies did not stain the plasma membrane where K\textsubscript{ATP} channels associated with cell depolarization would be localized. No specific GIP and Kir 6.1 colocalization was observed (Fig. 6C). This observation is consistent with our previous results that showed that none of 10 independently derived GIP-producing cell lines contained detectable levels of Kir 6.1 transcripts (39). Conversely, paraffin-embedded sections of mouse heart stained strongly with the same Kir 6.1 antibodies (Fig. 6A), and this staining was abolished if the antibodies were preincubated with the peptide used to immunize the rabbits (data not shown).

\textit{Complex pattern of Kir 6.2 expression in other EE cell populations.}\n
The pattern of Kir 6.2 expression in the intestine suggested that this K\textsubscript{ATP} subunit is expressed in subpopulations of EE cells. Therefore, additional double-label immunohistochemical studies were performed using goat anti-Kir 6.2 antibodies plus rabbit...
antibodies directed against a host of individual EE cell products. Several selected examples of the staining patterns are shown in Fig. 7, and a quantitative presentation of all of the results is presented in Table 3. Like GIP, the incretin hormone GLP-1 is secreted immediately after ingestion of a meal. We were unable to identify a single GLP-1-positive L cell that was also positive for Kir 6.2 (Table 3). Similarly, 100 and 99.3% of the cells that stained positive for CCK and SST, respectively, did not express Kir 6.2 (data not shown). In stark contrast, 98% of the cells that expressed CGA also expressed Kir 6.2 (Fig. 7, A–D). In the intestinal epithelium, SP is expressed predominantly in the crypts, and 95% of these cells also expressed Kir 6.2. Of the few SP-positive cells that were located on the villi, ~50% stained positive for Kir 6.2. Both secretin (Fig. 7, E–H) and serotonin-producing EE cells exhibited an intermediate phenotype with 57 and 36% of each population, respectively, also expressing Kir 6.2.

Secretion from GIP/Ins cells is both dependent and independent of L-type calcium channels. The preceding results suggest that gut K cells do not express plasma membrane-associated KATP channels and that secretion may be completely independent of the cytoplasmic ATP-to-ADP ratio. Because intracellular calcium is required for exocytosis, experiments were conducted to determine whether L-type calcium channels participate in hormone secretion by GIP/Ins cells. It has been reported that protein kinase C (PKC) can activate L-type calcium channels (16, 54, 56, 61, 63, 72). Thus the effects of phorbol 12-myristate 13-acetate (PMA) on both GIP and insulin secretion by GIP/Ins cells were examined. As shown in Figs. 1 and 8, addition of 10^{-6} M PMA to GIP/Ins clones 10 and 12 resulted in a fivefold increase in insulin release from GIP/Ins cells. This increase is similar to that observed after addition of meat hydrolysate, one of the most potent secretagogues that we have identified to date for GIP/Ins cells (39). Next, we examined the effects of verapamil, an inhibitor of L-type calcium channels, on PMA-stimulated insulin release from GIP/Ins cells. As shown in Fig. 8, increasing concentrations of verapamil inhibited insulin secretion from GIP/Ins cells. However, even 0.3 mM verapamil inhibited secretion by only ~50%. In contrast, 0.03 mM verapamil almost completely inhibited KCl-stimulated insulin release from the GIP/Ins cells. As with PMA, verapamil only partially prevented bombesin-stimulated insulin release (data not shown). Similar results were obtained when GIP release was determined (Fig. 9), indicating that these results are not an artifact of measuring insulin, rather than GIP release, from GIP/Ins cells. These results indicate that, depending on the secretagogue, calcium mobilization can occur via verapamil-sensitive and -insensitive calcium channels or from intracellular stores.

Ryanodine receptors (RyRs) mobilize calcium from intracellular stores and are present in β-cells (19). RyRs can be activated by 1.5 mM IBMX, caffeine, or theophylline. Consistent with this, addition of IBMX to MIN6 cells resulted in a fivefold increase in insulin release (Fig. 3). In contrast, addition of IBMX, either alone or along with glucose, had no effect on insulin release by GIP/Ins clone 10 or clone 12 cells (Fig. 1). F-1,6-P_2 activates RyRs, and, as shown in Tables 1 and 2, the intracellular concentration of this glycolytic intermediate increased 143-fold in GIP/Ins cells treated with 25 mM glucose. Taken together, these results strongly suggest that RyRs do not play a major role in hormone release from GIP/Ins cells.

Although somewhat controversial, intracellular glucolate has been proposed to represent an important secretagogue for β-cells (25, 26, 71). However, glucolate levels were increased similarly in GIP/Ins and MIN6 cells after addition of glucose and actually decreased after addition of glyceraldehyde or methyl
Pyruvate (Tables 1 and 2). Methyl glutamate, a cell-permeable form of glutamate, failed to stimulate insulin release from GIP/Ins cells (Fig. 1) and had very little effect on MIN6 cells (Fig. 3). Furthermore, addition of glucose plus glutamine, which profoundly increases intracellular glutamate levels (25), had no effect on secretion by MIN6 (Fig. 3) or GIP/Ins (data not shown) cells. Extracellular glutamate is a secretagogue for islet β-cells and increased insulin release from MIN6 cells >15-fold. In contrast, addition of glutamate with or without glucose failed to stimulate insulin release from GIP/Ins cells (data not shown). These results argue against intracellular or extracellular glutamate representing a secretagogue in K cells.

**DISCUSSION**

Although both islet β- and gut K cells secrete hormones in response to similar nutrients, peptide release by gut K cells appears to be K<sub>ATP</sub> channel independent. This conclusion is based on several convergent observations. First, cytoplasmic ATP/ADP regulates plasma membrane K<sub>ATP</sub> channel activity. Addition of either glucose or methyl pyruvate to GIP/Ins cells resulted in similar increases in the intracellular ATP-to-ADP ratio, but only methyl pyruvate stimulated hormone release. It should also be noted that metabolism of methyl pyruvate generates ATP in the mitochondria (31). Thus the measured ATP-to-ADP ratio represents the maximal ratio present in the cell cytoplasm, and...
the ATP-to-ADP ratio in GIP/Ins cells treated with glucose is probably greater than that in methyl pyruvate-treated cells. Second, the ATP-to-ADP ratio is nearly three times higher in GIP/Ins cells treated with 25 mM glucose vs. glyceraldehyde. However, glyceraldehyde, but not glucose, stimulated hormone release from GIP/Ins cells. Third, RT-PCR analysis of RNA samples prepared from 10 independently derived GIP-producing cell lines demonstrated that these cells express much lower levels of Kir 6.2 and SUR 1 than MIN6 insulinoma cells (39). Furthermore, none of the 10 cell lines expressed detectable levels of Kir 6.2 positive cells (red). The number of cells that expressed a specific hormone alone (e.g., GIP alone) and the number of hormone positive cells that also expressed Kir 6.2 (e.g., GIP + Kir 6.2) are listed. The % of cells is derived from the ratio of single or double-labeled cells to the total number of counted cells for a specific hormone multiplied by 100. Values for SP are for cells located only in the crypts.

Table 3. Expression of Kir 6.2 in specific subpopulations of EE cells

<table>
<thead>
<tr>
<th>No. of Cells</th>
<th>% Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>GIP alone</td>
<td>298</td>
</tr>
<tr>
<td>GIP + Kir 6.2</td>
<td>8</td>
</tr>
<tr>
<td>GLP-1 alone</td>
<td>304</td>
</tr>
<tr>
<td>GLP-1 + Kir 6.2</td>
<td>0</td>
</tr>
<tr>
<td>CCK alone</td>
<td>206</td>
</tr>
<tr>
<td>CCK + Kir 6.2</td>
<td>0</td>
</tr>
<tr>
<td>SST alone</td>
<td>302</td>
</tr>
<tr>
<td>SST + Kir 6.2</td>
<td>2</td>
</tr>
<tr>
<td>CGA alone</td>
<td>6</td>
</tr>
<tr>
<td>CGA + Kir 6.2</td>
<td>294</td>
</tr>
<tr>
<td>SP alone</td>
<td>13</td>
</tr>
<tr>
<td>SP + Kir 6.2</td>
<td>237</td>
</tr>
<tr>
<td>Secretin alone</td>
<td>170</td>
</tr>
<tr>
<td>Secretin + Kir 6.2</td>
<td>130</td>
</tr>
<tr>
<td>Serotonin alone</td>
<td>116</td>
</tr>
<tr>
<td>Serotonin + Kir 6.2</td>
<td>211</td>
</tr>
</tbody>
</table>

GIP, glucose-dependent insulinotropic polypeptide; Kir, inwardly rectifying potassium; GLP, glucagon-like peptide; CCK, cholecystokinin; SST, somatostatin; CGA, chromogranin A; SP, substance P; EE, enteroendocrine. Single paraffin sections of mouse small intestine were incubated with goat antibodies to Kir 6.2 plus rabbit antibodies to the indicated EE cell product followed by labeled secondary antibodies as described in Fig. 4. Single cells expressing a specific gut endocrine cell hormone were identified in a fluorescent microscope with filters designed to detect only FITC-conjugated secondary antibodies (green). The filters were then switched to identify Kir 6.2 positive cells (red). The number of cells that expressed a specific hormone alone (e.g., GIP alone) and the number of hormone positive cells that also expressed Kir 6.2 (e.g., GIP + Kir 6.2) are listed. The % of cells is derived from the ratio of single or double-labeled cells to the total number of counted cells for a specific hormone multiplied by 100. Values for SP are for cells located only in the crypts.

Fig. 6. Kir 6.1 expression in murine heart and small intestine. Heart (A) and small intestine (B and C) were treated as described in Fig. 4. Single sections were then stained with antibodies to Kir 6.1 (red) and/or GIP (green). Nuclei were visualized by staining with bisbenzimide (blue). Preincubation of the Kir 6.1 antibodies with the peptide used to generate the antibodies abolished all staining of Kir 6.1 (data not shown). Exposures in A and B are identical so that the relative levels of Kir 6.1 protein expression in the two different tissues could be compared. C is the same as B except GIP staining (green areas indicated by arrowheads) was included.
GIP/Ins cells do not express $K_{\text{ATP}}$ channels or the channels are incredibly distinct from those expressed by other tissues. It is possible that low levels of $K_{\text{ATP}}$ channels play a role in maintaining GIP/Ins cells in a hyperpolarized state rather than in allowing cells to depolarize. However, diazoxide had no effect on basal GIP or insulin secretion from GIP/Ins cells (unpublished observation). This result would argue against this possibility. Fifth, if the GIP-producing cell lines reflect what occurs in vivo, one would predict that K cells do not express $K_{\text{ATP}}$ channels. Double-label antibody staining clearly demonstrated that K cells in vivo do not express detectable levels of Kir 6.1 or Kir 6.2. Our antibodies against SUR 1 and 2 did not stain either small intestine or positive control tissues. Thus it is unknown whether these proteins are expressed in gut K cells in vivo. However, because functional $K_{\text{ATP}}$ channels require both SUR and Kir subunits and nei-

Fig. 7. Kir 6.2 expression in chromogranin A (CGA)- and secretin-producing enteroendocrine (EE) cells. Samples of mouse small intestine were stained as described in Fig. 4 except sections were incubated with goat anti-Kir 6.2 antibodies (red) plus rabbit anti-CGA (green; A–D) or rabbit anti-secretin (green; E–H). Black arrows with white outlines point to cells that coexpress Kir 6.2 plus CGA (D) or secretin (H). In H, arrowhead points to secretin-producing cells that do not coexpress Kir 6.2, and solid white arrows point to cells that express Kir 6.2 but not secretin.
ther Kir 6.1 nor Kir 6.2 are present in gut K cells, K\textsubscript{ATP} channels may not be expressed in gut K cells. In a previous report (39), we demonstrated that, unlike gut K cells in vivo, GIP/Ins cells do not secrete hormones in response to glucose, and it was hypothesized that this major GIP “secretagogue” does not act directly on gut K cells. Rather, glucose uptake and metabolism by adjacent enterocytes is required for glucose-stimulated GIP release by K cells (see Ref. 39 for detailed discussion). This hypothesis is consistent with the results presented in this paper, since glucose is rapidly metabolized by GIP/Ins cells even though it does not stimulate hormone release. Furthermore, K cells in vivo do not express detectable levels of Kir 6.1 or Kir 6.2, and hormone release from GIP-producing cell lines is independent from the increased ATP-to-ADP ratio that is generated via a high rate of glucose metabolism. EE cells that produce GLP-1, CCK, and SST also release hormones in response to nutrients. It is interesting to note that very few individual cells that expressed any of these hormones also expressed detectable levels of Kir 6.2. Therefore, nutrient sensing by four different subpopulations of EE vs. islet \beta-cells appears to occur via distinct mechanisms (see below). However, GIP/Ins cells secreted GIP and insulin after addition of 30 mM KCl, suggesting that ATP-independent potassium channels play a role in depolarizing gut K cells. In support of this hypothesis, it has been reported that parent STC-1 cells express at least two types of ATP-independent potassium channels (58).

Although not expressed in gut K cells, Kir 6.2 was present in specific subpopulations of EE cells. Most notably, nearly all of the EE cells that expressed CGA or SP also expressed Kir 6.2. This suggests that secretion of CGA and SP immunoreactive hormones and GIP is regulated by distinct mechanisms. More surprising was the observation that only 43 or 64% of secretin- or serotonin-producing cells, respectively, coexpressed Kir 6.2. This raises the possibility that secretion of these hormones is regulated by ATP/ADP-sensitive and ATP/ADP-insensitive mechanisms. Alternatively, SP- and secretin-producing EE cells that also express Kir 6.2 may contain combinations of hormones that are distinct from those that are Kir 6.2 negative. In either case, Kir 6.2 expression can be used to further define specific subpopulations of gut endocrine cells.

On the basis of results using a Simian virus (SV) 40 T antigen-transformed cell line, it has been suggested that glucose stimulates secretion of GLP-1 from gut L cells via closure of K\textsubscript{ATP} channels (40). However, comparison of results in Figs. 1, 2, and 6 of that paper reveal that there is no correlation between the effects of glucose or tolbutamide on the firing of action potentials and GLP-1 secretion. Furthermore, tolbutamide stimulated secretion only \~2-fold, whereas glucose plus IBMX and forskolin stimulated secretion \~15-fold. Thus, at best, K\textsubscript{ATP} channels play a minor role in regulating secretion by gut L cells. This conclusion is consistent with our in vivo immunohistochemical studies indicating that gut L cells, like gut K cells, do not express detectable Kir 6.1 or Kir 6.2. Patch-clamp studies have also demonstrated the presence of K\textsubscript{ATP} channels in parent STC-1 cells (2, 28, 30, 58). Thus it has been proposed that they play a role in regulating CCK secretion (2, 27, 28). However, the results presented in this paper suggest that K\textsubscript{ATP} channels play, at best, a very minor role in regulating CCK secretion, since CCK-producing cells in vivo do not express detectable levels of Kir 6.1 or Kir 6.2. STC-1 is a heterogeneous hormone-producing cell line, and the previous studies most likely measured K\textsubscript{ATP} channels in CGA-, secretin-, SP-, or serotonin-expressing cells, since these cells express Kir 6.2 in vivo. STC-1 cells exhibit at least three types of potassium channel activities (58) and...
also express L-type calcium channels. Bombesin, sodium oleate, phenylalanine, barium chloride, and PMA are all potent CCK secretagogues for STC-1 cells (6, 29, 30, 58, 59), whereas glucose is a very weak stimulant (27). Inhibition of L-type calcium channel activity prevented secretagogue-stimulated CCK release. Thus, as with gut K cells, secretagogues probably stimulate CCK release by their ability to mobilize calcium independently of activation of \( K_{ATP} \) channels. Because SST-producing cells do not express Kir 6.1 or Kir 6.2 in vivo, it seems likely that EE cell secretion of this hormone is also \( K_{ATP} \) channel independent.

The results of these studies raise the question as to what controls calcium mobilization and thus hormone secretion from not only gut K cells but also the other nutrient-responsive EE cell populations. Evidence presented in this paper supports the notion that the RyRs are not involved in calcium mobilization by GIP-producing cells. Verapamil-sensitive L-type calcium channels play a role in regulating secretion by GIP/Ins cells since KCl-stimulated hormone release was completely blocked by verapamil. However, verapamil-insensitive mechanisms also play an important role in regulating hormone release from GIP/Ins cells, since this drug inhibited PMA- and bombesin-stimulated insulin and GIP release by only ~50%. That verapamil inhibited meat hydrolysate-stimulated hormone release from GIP/Ins cells (39) suggests that protein hydrolysates may be directly depolarizing GIP/Ins cells, as was observed after addition of KCl. Bombesin binds to the GIP receptor and activates phospholipase C, resulting in increases in inositol phosphate, diacylglycerol, and intracellular calcium levels (60). Thus bombesin could potentially mobilize calcium via activation of PKC as well as from intracellular stores via inositol 1,4,5-trisphosphate receptors (IP3Rs). IP3Rs are expressed in secretory granules of neuroendocrine cells and are thought to facilitate secretion by controlling calcium release from the granules (3). There are at least three different IP3Rs in mammalian cells, and studies are currently underway to determine whether any of these isoforms are expressed in gut K cells in vivo.

Results presented in this and our previous study (39) have provided unexpected insights into not only gut K cell physiology but also the regulation of secretion by other EE cell populations. Insulin and GIP are released from islet \( \beta \)- and gut K cells, respectively, in response to similar nutrients immediately after ingestion of a meal (5, 52, 64, 66). This is not surprising, since GIP potentiates glucose-stimulated insulin release from \( \beta \)-cells. Thus GIP actually lies upstream of insulin in terms of regulating glucose homeostasis. EE and islet \( \beta \)-cells are both derived from the primitive gut endoderm and express many of the same transcription factors and processing enzymes (23, 36, 37, 55). In fact, transgenes expressed using the rat insulin promoter (RIP) are frequently expressed in EE cells and in islet \( \beta \)-cells. For example, the STC-1 EE cell line, from which the GIP/Ins cells were derived, was isolated from a murine intestinal carcinoma that arose in a double-transgenic mouse generated by crossing RIP/SV40 T antigen and RIP/Polyoma small T antigen mice (43). The intimate relationship between EE and islet \( \beta \)-cells is further illustrated by the fact that, in some lower vertebrates that lack islets but contain a brain-gut axis, insulin is produced and secreted by gut endocrine cells (9). Thus it is quite surprising that gut K cells do not appear to express \( K_{ATP} \) channels and seem to sense glucose in a distinct fashion from islet \( \beta \)-cells. Similarly, why don’t other nutrient-responsive gut endocrine cell populations that produce and secrete GLP-1, SST, and CCK express \( K_{ATP} \) channels? On the other hand, why do EE cells that express CGA immunoreactive peptides express Kir 6.2 as do islet \( \beta \)-cells? Although these are philosophical questions, it is clear that GIP and insulin secretion from gut K cells and islet \( \beta \)-cells, respectively, occurs via very distinct mechanisms. Because of the roles for GIP in maintaining blood glucose homeostasis and promoting obesity, it is important to understand the molecular mechanisms that regulate hormone production and secretion by gut K cells.

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REGULATION OF SECRETION BY ENTEROENDOCRINE CELLS


