Synergistic Potent Insulin Release by Combinations of Weak Secretagogues in Pancreatic Islets and INS-1 Cells*

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Insulin secretion by the beta cell depends on anaplerosis in which insulin secretagogues are metabolized by mitochondria into molecules that are most likely exported to the extramitochondrial space where they have signaling roles. However, very little is known about the products of anaplerosis. We discovered an experimental paradigm that has begun to provide new information about these products. When various intracellular metabolites were applied in combination to overnight-cultured rat or human pancreatic islets or to INS-1 832/13 cells, they interacted synergistically to strongly stimulate insulin release. When these same metabolites were applied individually to these cells, insulin stimulation was poor. Discerning the contributions of the individual compounds to metabolism has begun to allow us to dissect some of the pathways involved in insulin secretion, which was not possible from studying individual secretagogues.

Monomethyl succinate (MMS) combined with a barely stimulatory concentration of α-ketoisocaproate (KIC) (2 mM) stimulated insulin release in cultured rat islets 18-fold versus 21-fold for 16.7 mM glucose. MMS plus low glucose (2 mM) or pyruvate (5 mM) gave 11- and 9-fold stimulations. These agents also potentiated MMS-induced insulin release in fresh islets, and KIC plus MMS gave synergistic insulin release in cultured human islets. In INS-1 cells, neither MMS nor KIC (10 mM) was an insulin secretagogue, but when added together KIC (2 mM) and MMS stimulated insulin release 7-fold (versus 12-fold for glucose). In islets and INS-1 cells, conditions that stimulated insulin release caused large relative increases in acetoacetate, which is a precursor of pathways to short chain acyl-CoAs. Liquid chromatography-tandem mass spectrometry measurements of acetyl-CoA, acetoacetyl-CoA, succinyl-CoA, hydroxymethylglutaryl-CoA, and malonyl-CoA confirmed that they were increased by insulin secretagogues. The results suggest a new mechanism of insulin secretion in which anaplerosis increases short chain acyl-CoAs that have roles in insulin exocytosis.

The metabolism of all fuel insulin secretagogues depends on mitochondria. Besides ATP production, the net synthesis by beta cell mitochondria of citric acid cycle intermediates (anaplerosis) (1) is important for the stimulation of insulin secretion (2–13). The rate of anaplerosis in the pancreatic beta cell is especially high. For example, glucose, the most potent physiologic insulin secretagogue, stimulates insulin secretion by its metabolism through aerobic glycolysis. The terminal metabolite of glycolysis, pyruvate, then enters mitochondria, where 50% of it is carboxylated into oxaloacetate by the anaplerotic enzyme pyruvate carboxylase (2–7). The amount of this enzyme is as high in the pancreatic beta cell as in gluconeogenic tissues (6), such as liver, but the beta cell does not support gluconeogenesis (2). When the oxaloacetate condenses with acetyl-CoA derived from the decarboxylation of pyruvate, the concentration of any citric acid cycle intermediate can be increased. Although it is widely believed that anaplerosis plays an important role in insulin secretion, surprisingly little is known about which intermediates are important or what the intra- or extramitochondrial directions of their flux are subsequent to their synthesis.

In an effort to explain part of the requirement for the high rate of anaplerosis in insulin secretion, we proposed the “succinate mechanism” of insulin release (2, 14). This hypothesis used our own data and data from the literature to suggest why methyl esters of succinic acid have consistently been found to be the only insulinotropic esters of any esters of a citric acid cycle intermediate tested, or the most insulinotropic, depending on the individual study. The current work takes advantage of an interesting idiosyncrasy of succinic acid monomethyl ester-induced insulin release to further delve into the “succinate mechanism,” which, in its more developed form, suggests that changes in the levels of several short chain acyl-CoAs, such as acetoacetyl-CoA, hydroxymethylglutaryl-CoA, and succinyl-CoA or closely related intermediates, are necessary for insulin secretion (2).

As we first showed and others have confirmed in many studies, methyl esters of succinate are relatively potent stimulators of insulin release in freshly isolated rat pancreatic islets (3–6, 15–22). The esters are hydrolyzed to succinate, a citric acid cycle intermediate that is a classical mitochondrial energizer that has been used for decades in mitochondrial studies. Within the mitochondria, succinate can be directly converted to four other citric acid cycle intermediates, succinyl-CoA, fumarate, malate, and oxaloacetate (Fig. 1, reactions 21 and 7–10). In addition, in freshly isolated pancreatic islets, malate or fumarate can exit the mitochondria and be converted to pyruvate via malic enzyme in the cytosol (Fig. 1, reactions 8, 9, 22, and 12). The succinate-derived carbon can then reenter mitochondria as pyruvate (2, 6). The pyruvate can be decarboxylated by pyruvate dehydrogenase to form acetyl-CoA. Acetyl-CoA can be converted to citrate (Fig. 1, reactions 1 and 3), and its acetal component can be metabolized to two CO₂ molecules in the citric acid

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Synergistic Insulin Release by Nonsecretagogue Combinations

Chris Newgard (29). Human pancreatic islets were from Bernhard J. Hering (University of Minnesota). Succinic acid monomethyl ester and other chemicals were from Sigma.

Pancreatic Islet Tissue Culture—Islets were isolated from 200–250-g fed rats by collagenase digestion as previously described (3–6). Islets were maintained overnight in RPMI 1640 tissue culture medium modified to contain 5 mM glucose instead of 11.1 mM glucose. Other media (mentioned under “Results”) were used unmodified. All tissue culture media used for overnight culture contained 10% fetal bovine serum as well as penicillin (100 units/ml) and streptomycin (100 µg/ml).

Insulin Release—Insulin release from pancreatic islets was studied as previously described (15–19). INS-1 832/13 cells were cultivated as monolayers in tissue culture medium that is standard for cultivating INS-1 cells (RPMI 1640 tissue culture medium (contains 11.1 mM glucose) supplemented with 10% fetal bovine serum, 1 mM sodium pyruvate, 50 µM β-mercaptoethanol, and 10 mM Hepes buffer (30) with 100 units/ml penicillin and 100 µg/ml streptomycin in 24-well tissue culture plates. For 22 h prior to an insulin release experiment, the medium was modified to contain 5 mM glucose. For the 2 h immediately before insulin release was studied, the cells were maintained in Krebs-Ringer bicarbonate buffer containing 15 mM Hepes and 15 mM NaHCO₃ (with the NaCl concentration adjusted to maintain osmolality at 300–310 mOsM), 0.5% bovine serum albumin, and 3 mM glucose (29). Plates were then washed once with the Krebs-Ringer Hepes bovine serum albumin solution containing no glucose, and 1 ml of this solution containing test compounds was added to each well to study insulin release. After 1 h, samples of incubation solution were collected and centrifuged to sediment any cells potentially floating in the incubation solution. Part of the supernatant fraction was removed and saved for insulin measurements by radioimmunoassay. The plates were then washed once with Krebs-Ringer solution containing no added protein, water was added to the plates, and the mixture containing the lysed cells was removed and saved for estimation of total cellular protein.

Insulin release from human islets was studied 1 day after they were isolated from the donor and shipped overnight in CMRL tissue culture medium containing 10% fetal bovine serum. The islets were washed and maintained for 2 h at 37 °C in RPMI 1640 medium modified to contain 5 mM glucose and 10% fetal bovine serum before they were washed again, and insulin release was measured in Krebs-Ringer bicarbonate Hepes solution containing 0.5% bovine serum albumin.

Metabolite Measurements—Rat pancreatic islets were incubated in batches of 100/test tube for 30 min at 37 °C in 200 µl of Krebs-Ringer bicarbonate buffer, pH 7.3, containing secretagogues. The Krebs-Ringer solution was quickly removed, and 50 µl of 6% perchloric acid was added to the islet pellet. The islet pellet was vortexed vigorously and centrifuged. The perchloric acid supernatant fraction was removed and neutralized to pH ~7 with about 14 µl of 15% KOH per 50 µl of extract. The perchloric acid pellet was saved for measurement of total cellular protein. Metabolites in 5–20 µl of neutralized extract were measured by alkali-enhanced fluorescence as previously described in detail (11). Fluorescence in blank samples contain-

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**EXPERIMENTAL PROCEDURES**

Materials—Sprague-Dawley rats were from Harlan Sprague-Dawley (Madison, WI). The INS-1 832/13 cell line was from

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2 The abbreviations used are: KIC, α-ketoisocaproate; LC, liquid chromatography; MS, mass spectrometry; ESI, electrospray ionization; HPLC, high pressure liquid chromatography; MMS, monomethyl succinate.
after 30 min at 37 °C, this solution was quickly withdrawn, 2 ml mM sodium Hepes buffer, pH 7.3. Secretagogues were added to be performed, this medium was replaced with fresh medium described above. Twenty-four hours before an experiment was done, cells were washed two times in phosphate-buffered saline and once in Krebs-Ringer bicarbonate buffer and incubated in 0.2 ml of Krebs-Ringer bicarbonate solution modified to contain 15 mM NaHCO₃, 15 mM Hepes buffer, pH 7.3, and 0.5% bovine serum albumin. There was about 0.5 mg of cell protein/test tube. Metabolism was stopped after 30 min by adding 0.1 ml of 6% perchloric acid. The mixture was centrifuged, and the perchloric acid extract was removed and neutralized to about pH 7 with about 13 μl of 30% KOH. Blanks containing all additions except cells were treated identically to samples containing cells, and background values for a metabolite or protein (bovine serum albumin) were subtracted from the values of samples containing cells. This occasionally gave a negative value for a metabolite when its level was low. Metabolites were also studied in monolayers of INS-1 832/13 cells maintained on 100-mm tissue culture plates in the Krebs-Ringer 0.5% bovine serum albumin solution. The reaction was stopped after 30 min by adding 1 ml of 6% perchloric acid to each plate, and the cells were processed for metabolite measurements exactly as described in Ref. 11. Since the levels of the metabolites were similar (the values from the cells in monolayers were about 30% lower than from cells in suspension, but the -fold changes in the presence of specific secretagogues were similar), the combined results from the two methods of incubation are shown in Tables 7 and 8.

Extraction of Short Chain Acyl-CoAs—To extract short chain CoAs from cells and to prepare them for LC-MS/MS, we liberally modified a procedure used by the Brunengraber laboratory (32). INS-1 832/13 cells were maintained on 150-mm diameter plates in the modified RPMI 1640 tissue culture medium described above. Twenty-four hours before an experiment was to be performed, this medium was replaced with fresh medium modified to contain 5 mM glucose. On the day of the experiment, plates were washed twice with 10 ml of phosphate-buffered saline and once with 10 ml of Krebs-Ringer bicarbonate solution modified to contain 15 mM sodium bicarbonate and 15 mM sodium Hepes buffer, pH 7.3. Secretagogues were added to the plates in 10 ml of the modified Krebs-Ringer solution, and after 30 min at 37 °C, this solution was quickly withdrawn, 2 ml of 1% trifluoroacetic acid and 50% methanol was added to each plate, and the plates were placed at −80 °C. After 5–10 min at −80 °C, cells were scraped off the plates. Each plate was washed with 1 ml of 50% methanol, and the wash was combined with the cellular material. The mixtures were vortexed vigorously, and 60 μl of the suspension was removed and saved for protein estimation (by the Lowry method (6, 11)); then the mixtures were centrifuged to remove protein. The supernatant fractions were evaporated to dryness in a SpeedVac concentrator (Savant, Farmingdale, NY) in preparation for solid phase extraction. Dried samples were reconstituted in 1 ml of 0.1% trifluoroacetic acid, vortexed, and kept on ice until ready for extraction. Samples were individually applied to preconditioned and equilibrated 3-ml Oasis® HLB cartridges (Waters, Milford, MA). The cartridges were washed with 3 ml of 0.1% trifluoroacetic acid and dried by pushing a small volume of air through the cartridge to remove any residual liquid. The short chain acyl-CoAs were eluted in two phases, first by applying 0.75 ml of 70% methanol to the cartridge and reapplying the eluate to the cartridge again to elute a less hydrophobic fraction and then with 0.75 ml of 100% methanol also applied twice to the cartridge to elute a more hydrophobic fraction. Both the 70% methanol eluate and the 100% methanol eluate were evaporated to dryness, dissolved in 50 μl of 0.05% trifluoroacetic acid, and analyzed separately by LC-MS/MS. Values from the 70% methanol and 100% methanol eluates were added together to provide the data shown in Table 9.

LC-ESI-MS/MS Analysis of CoA Compounds—LC-ESI-MS/MS analysis of CoA thioesters extracted from INS-1 cells was carried out on an LC-MS/MS system consisting of an Agilent 1100 series liquid chromatograph (Palo Alto, CA), including a vacuum degasser, a quaternary pump, and an autosampler, coupled with an Applied Biosystems 3200 QTRAP tandem mass spectrometer (Foster City, CA) equipped with a Turbo V™ ion spray source. Chromatography separations were done on a 1.0 × 150-mm Inertsil ODS-3 (5-μm particles, 100-Å pores) end-capped reversed phase column onto which 40 μl of each extraction was automatically loaded. HPLC-delivered solvents were as follows: A, 0.05% (v/v) trifluoroacetic acid in water; B, 90% (v/v) acetonitrile, 0.05% (v/v) trifluoroacetic acid at 0.050 ml/min. After a 30-min equilibration at 100% buffer A, the CoA thioesters were eluted directly into the electrospray orifice over a 30-min 2% (v/v) B to 99% (v/v) B gradient and 5 min at 99% (v/v) B hold. As metabolites eluted from the HPLC column into the electrospray source, the multiple reaction monitoring mode selectively measured spectra for five CoA thioesters based on their precursor and specific fragment mass (acetyl-CoA m/z = 810.3, fragment m/z = 303.2; acetocetyl-CoA m/z = 852.3, fragment m/z = 345.2; malonyl-CoA m/z = 854.3, fragment m/z = 347.2; succinyl-CoA m/z = 868.3, fragment m/z = 361.2; 3-hydroxy-3-methylglutaryl-CoA m/z = 912.3, fragment m/z = 405.4). Tolerance for the molecular mass of precursor was at a unit mass resolution. To quantify various concentrations of the acyl-CoA thioesters, all five CoA thioester standards were mixed together and were run immediately before and after the biological samples to generate standard curves. These were used to determine the concentrations of CoA thioesters in the samples by manual or automatic extrapolation from the peak.
Synergistic Insulin Release by Nonsecretagogue Combinations

Pancreatic islets were isolated from fed Sprague-Dawley rats weighing 200 g and were maintained for 22 h in RPMI 1640 tissue culture medium modified to contain 5 mM glucose and 10% fetal calf serum. Islets were then washed carefully and incubated with various agents in Krebs-Ringer bicarbonate buffer, pH 7.4, containing 0.5% bovine serum albumin for 1 h to study insulin release. Results are expressed as the mean ± S.E. with the number of replicate incubations in parentheses.

Synergistic Insulin Release from Methyl Succinate Combined with Substimulatory KIC or Glucose or the Nonsecretagogue Pyruvate in Cultured Rat Pancreatic Islets

**TABLE 1**

| Incubation condition | Insulin release | Relative insulin release |
|----------------------|-----------------|-------------------------|
|                      | microunits insulin/5 islets/1 h | × control |
| No addition          | 13 ± 2 (29)      | Control                 |
| MMS (10 mM)          | 12 ± 1 (27)      | 1                       |
| KIC (2 mM)           | 26 ± 2 (17)a     | 2                       |
| MMS (2 mM) + KIC (2 mM) | 52 ± 2 (6)b   | 4                       |
| MMS (5 mM) + KIC (2 mM) | 155 ± 9 (6)b  | 12                      |
| MMS (10 mM) + KIC (2 mM) | 228 ± 17 (17)b | 18                      |
| Pyruvate (5 mM)      | 15 ± 3 (11)      | 1                       |
| MMS (10 mM) + pyruvate (5 mM) | 121 ± 6 (9)a | 9                       |
| Glucose (10 mM)      | 19 ± 4 (9)       | 1.5                     |
| MMS (10 mM) + glucose (2 mM) | 144 ± 24 (9)a | 11                      |
| Glucose (16.7 mM) (positive control) | 271 ± 18 (17)a | 21                      |
| KIC (10 mM) (positive control) | 304 ± 27 (12)a | 24                      |

* p < 0.001 versus no addition control or MMS alone.

**TABLE 2**

| Incubation condition | Insulin release |
|----------------------|-----------------|
|                      | microunits insulin/5 islets/1 h |
| No addition          | 13 ± 2 (29)     |
| MMS (10 mM)          | 12 ± 1 (27)     |
| Glucose (16.7 mM)    | 271 ± 18 (17)a  |
| Leucine (2 mM)       | 9 ± 2 (12)      |
| MMS (10 mM) + leucine (2 mM) | 12 ± 1 (12)    |
| Leucine (10 mM)      | 14 ± 2 (12)     |
| Leucine (10 mM) + KIC (2 mM) | 60 ± 4 (6)a   |
| Acetyl carnitine (1 mM) | 20 ± 4 (9)   |
| MMS (10 mM) + acetyl carnitine (1 mM) | 21 ± 5 (9)    |
| Acetyl carnitine (5 mM) | 18 ± 3 (11)   |
| MMS (10 mM) + acetyl carnitine (5 mM) | 41 ± 4 (10)a  |
| Acetoacetate (5 mM)  | 10 ± 1 (12)     |
| MMS (10 mM) + acetoacetate (5 mM) | 49 ± 5 (17)a  |
| Acetate (5 mM)       | 18 ± 4 (12)     |
| MMS (10 mM) + acetate (5 mM) | 35 ± 4 (12)a  |

* p < 0.001 versus no addition or 10 mM methyl succinate.

RESULTS

Insulin Release—Islets maintained overnight in RPMI tissue culture medium containing 5 mM glucose did not respond to methyl succinate with insulin release (Table 1). We tested whether supplementing RPMI 1640 tissue culture medium with various combinations of agents or maintaining the islets for 22 h in various media instead of RPMI 1640 medium would permit methyl succinate by itself to stimulate insulin release. Maintaining islets overnight in NCTC-135 medium, Dulbecco’s minimal essential medium, or HY medium containing 10% fetal bovine serum or hCell SBMI 06 medium (hCell Technology Inc.) did not permit 10 mM methyl succinate to stimulate insulin release above that of the no addition control. The following additions or modifications to RPMI 1640 tissue culture medium containing 10% fetal bovine serum did not permit 10 mM methyl succinate to stimulate insulin release: 1 mM glucose, 5 mM pyruvate, 10 mM glutamine; 1 mM glucose, 5 mM pyruvate, 10 mM leucine; 5 mM pyruvate, 5 mM glucose, 5 mM malate; and 5 mM glucose, 1 mM sodium acetate, 1 mM pyruvate, 0.1 mM coenzyme A, 50 μM dithiothreitol, 0.5 mM NAD, 0.5 mM NADP (data not shown).

Table 1 shows that methyl succinate at 10 mM, a maximally stimulatory concentration for insulin release from freshly isolated rat pancreatic islets (3–6, 15–21), was completely ineffective in stimulating insulin release from rat pancreatic islets after they were maintained overnight in RPMI 1640 tissue culture medium containing 10% fetal bovine serum and modified to contain 5 mM glucose. However, when 10 mM methyl succinate was combined with 2 mM KIC, insulin release was increased 18-fold and was nearly as high as that observed with 16.7 mM glucose or 10 mM KIC, which are concentrations of these secretagogues that induce maximal stimuli for insulin release from fresh or cultured rat islets (Table 1). In the presence of 2 mM KIC alone, the insulin released was only double that observed without any addition to the medium. Glucose alone at 2 mM and pyruvate alone at 5 mM did not stimulate insulin release above the “no addition” control. However, similar to the effect of KIC at 2 mM, when 2 mM glucose or 5 mM pyruvate was combined with 10 mM succinate, insulin release was markedly increased, 11- and 9-fold, respectively. When methyl succinate was present at concentrations of 5 or 2 mM in combination with 2 mM KIC, insulin release was also significantly increased versus the no addition control but was lower than that observed in the presence of 10 mM methyl succinate plus 2 mM KIC (Table 1). It was previously shown that leucine does not stimulate insulin release from rat pancreatic islets maintained in tissue culture at 5 mM or higher glucose (19, 33, 34), and it did not by itself stimulate insulin release from cultured islets in the current study (Table 2). Although KIC is the first metabolite of leucine and 2 mM KIC interacted strongly with methyl succinate to stimulate a large amount of insulin release, interestingly leucine at 2 mM in combination with 10 mM methyl succinate permitted only weak stimulation of insulin release in cultured rat islets (Table 2). KIC, pyruvate, and glucose can each provide acetyl-CoA. Other fuels that might provide acetyl-CoA, such as acetate, acetoacetate, or acetyl carnitine, when added in combination with 10 mM methyl succinate gave insulin release 3–4-fold above the no addition control (Table 2).

Synergism between 10 mM methyl succinate and 2 mM KIC was also observed in human pancreatic islets maintained in tissue culture and in INS-1 cells (Table 3). Interestingly, neither 10 mM KIC alone, which is about as potent as glucose in stimulating insulin release from rat or mouse pancreatic islets, nor 10 mM methyl succinate alone stimulated insulin release from INS-1 832/13 cells. Pyruvate alone, at any concentration, is not an insulin secretagogue in fresh or cultured rat or mouse islets (24–28). However, there are previous reports of pyruvate stimulating insulin release from INS-1 cells (27, 35). Table 3 shows that pyruvate, even at 1 mM, was as potent as 11 mM glucose in areas using Applied Biosystems Analyst 1.4.1 data analysis software (Foster City, CA).

Data Analysis—Statistical significance was estimated by analysis of variance and confirmed with Student’s t test.

**TABLE 2**

| Incubation condition |
|----------------------|
|                      |
| No addition          |
| MMS (10 mM)          |
| Glucose (16.7 mM)    |
| Leucine (2 mM)       |
| MMS (10 mM) + leucine (2 mM) |
| Leucine (10 mM)      |
| Leucine (10 mM) + KIC (2 mM) |
| Acetyl carnitine (1 mM) |
| MMS (10 mM) + acetyl carnitine (1 mM) |
| Acetyl carnitine (5 mM) |
| MMS (10 mM) + acetyl carnitine (5 mM) |
| Acetoacetate (5 mM)  |
| MMS (10 mM) + acetoacetate (5 mM) |
| Acetate (5 mM)       |
| MMS (10 mM) + acetate (5 mM) |

**TABLE 3**

| Incubation condition |
|----------------------|
|                      |
| No addition          |
| MMS (10 mM)          |
| Glucose (16.7 mM)    |
| Leucine (2 mM)       |
| MMS (10 mM) + leucine (2 mM) |
| Leucine (10 mM)      |
| Leucine (10 mM) + KIC (2 mM) |
| Acetyl carnitine (1 mM) |
| MMS (10 mM) + acetyl carnitine (1 mM) |
| Acetyl carnitine (5 mM) |
| MMS (10 mM) + acetyl carnitine (5 mM) |
| Acetoacetate (5 mM)  |
| MMS (10 mM) + acetoacetate (5 mM) |
| Acetate (5 mM)       |
| MMS (10 mM) + acetate (5 mM) |

* p < 0.001 versus no addition or 10 mM methyl succinate.
TABLE 3
Synergistic effects of methyl succinate plus KIC in human pancreatic islets and in INS-1 cells

| Incubation condition | Human islet insulin release | Relative insulin release | \( \times \) control |
|----------------------|-----------------------------|--------------------------|---------------------|
| No addition          | 15 ± 2 (5)                  | Control                  | 1                   |
| MMS (10 mM)          | 45 ± 10 (6)\(^a\)          |                          | 3                   |
| KIC (2 mM)           | 43 ± 4 (6)\(^a\)           |                          | 3                   |
| MMS (10 mM) + KIC (2 mM) | 222 ± 26 (6)\(^b\)   |                          | 15                  |
| Glucose (16.7 mM)    | 632 ± 48 (5)\(^b\)         |                          | 42                  |

\( ^a \) \( p < 0.02 \) versus no addition, same cell type.
\( ^b \) \( p < 0.001 \) versus MMS or KIC alone, same cell type.

TABLE 4
Synergistic effects of methyl succinate plus KIC, glucose, or pyruvate on insulin release in freshly isolated rat pancreatic islets

| Incubation condition | INS-1 cell insulin release | Relative insulin release | \( \times \) control |
|----------------------|---------------------------|--------------------------|---------------------|
| No addition          | 1080 ± 70 (28)            | Control                  | 1                   |
| MMS (10 mM)          | 1080 ± 60 (16)            |                          | 1                   |
| KIC (2 mM)           | 1150 ± 60 (16)            |                          | 1                   |
| MMS (10 mM) + KIC (2 mM) | 1170 ± 70 (28)         |                          | 1                   |
| Glucose (11.1 mM)    | 7150 ± 730 (16)\(^b\)     |                          | 7                   |
| Pyruvate (1 mM)      | 12,850 ± 1230 (22)\(^b\) |                          | 12                  |
| Pyruvate (1 mM) + methyl succinate (10 mM) | 14,990 ± 1280 (12)\(^b\) |                         | 14                  |
| Pyruvate (5 mM)      | 13,990 ± 1500 (8)\(^b\)  |                          | 13                  |
|                      | 14,240 ± 980 (21)\(^b\)  |                          | 13                  |

\( ^b \) \( p < 0.001 \) versus no addition MMS alone, 2 mM KIC alone, or pyruvate alone.
\( ^b \) \( p < 0.025 \) versus MMS alone.

stimulating insulin release from INS-1 832/13 cells. Although methyl succinate by itself stimulates insulin release in freshly isolated rat islets, 2 mM KIC significantly potentiated methyl succinate-induced insulin release in freshly isolated rat pancreatic islets (Table 4). In addition, methyl succinate in combination with either 2 mM glucose or 5 mM pyruvate (not a secretagogue in islets) augmented insulin release significantly above that seen with methyl succinate alone (Table 4).

Metabolite Levels—Table 5 shows that 10 mM methyl succinate alone or in combination with 2 mM KIC raised malate about 30-fold in cultured pancreatic islets, as was shown previously for methyl succinate alone (11), and also increased citrate levels about 60%. KIC at 2 mM alone or 10 mM alone also increased citrate (about 30 and 70%, respectively). KIC (2 mM) plus methyl succinate lowered glutamate 30%, and 10 mM KIC lowered glutamate 32%, whereas 2 mM KIC lowered glutamate 16%. Methyl succinate raised aspartate 58%, whereas 10 mM KIC lowered aspartate 35%, and 2 mM KIC lowered aspartate 16%. The increases in malate and citrate and decrease in glutamate occurred in the presence of conditions that stimulate insulin release, such as the presence of 2 mM KIC plus methyl succinate and 10 mM KIC alone but also in the presence of methyl succinate alone, which does not stimulate insulin release in cultured pancreatic islets. This suggests that these changes in malate, citrate, and glutamate may be important but are not sufficient for insulin secretion.

Perhaps more informative in providing clues to which anaplerotic products might be important for insulin secretion were the changes in acetoacetate and \( \alpha \)-ketoglutarate (Table 6). In rat islets, acetoacetate was in general increased the most by combinations of methyl succinate plus KIC, glucose, or pyruvate or by 10 mM KIC alone, which are conditions that stimulated insulin release the most. Many of the additions to the islets also increased \( \alpha \)-ketoglutarate, and conditions that increased insulin release the most, such as 2 mM KIC plus MMS or 10 mM KIC alone, increased \( \alpha \)-ketoglutarate the most. However, both glucose and KIC (10 mM) alone stimulated insulin release out of proportion to the increases in \( \alpha \)-ketoglutarate. In addition, methyl succinate alone and pyruvate alone caused moderate increases in \( \alpha \)-ketoglutarate, but did not stimulate insulin release. Although glucose is the most potent physiologic insulin secretagogue, stimulatory glucose (16.7 mM) did not increase acetoacetate or \( \alpha \)-ketoglutarate as much as the other secretagogues. We have previously observed that glucose frequently causes only small or moderate increases in many metabolites in islets (11, 36). This might be because the metabolism of glucose is very efficient, such that production of glucose metabolites is closely balanced by their consumption.

In INS-1 832/13 cells, similarly to islets, conditions that increased malate and citrate and lowered glutamate the most, such as 11.1 mM glucose (a maximal insulin stimulus in INS-1 832/13 cells), 5 mM pyruvate alone, and 2 mM KIC plus 10 mM methyl succinate, were those that stimulated insulin release. Other conditions that changed the levels of these metabolites,
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TABLE 5
Effect of various insulin secretagogues on levels of some metabolites in cultured rat pancreatic islets

Islets were maintained for 22 h in RPMI 1640 medium modified to contain 5 mM glucose and 10% fetal bovine serum and then washed and incubated with insulin secretagogues in Krebs-Ringer bicarbonate buffer, pH 7.3. After 30 min, metabolism was stopped, and metabolites were extracted and assayed as described under “Experimental Procedures.” Results are the mean ± S.E. with the number of replicate incubations in parentheses.

| Secretagogue added (concentration) | Metabolite concentration |      |
|------------------------------------|--------------------------|------|
|                                    | Malate       | Citrate   | Glutamate    | Aspartate   |
|                                    | nmol metabolite/mg islet protein |       |              |             |
| None                               | 0.50 ± 0.1 (7) | 4.8 ± 0.3 (7) | 23.4 ± 2.0 (11) | 12.2 ± 0.6 (8) |
| KIC (2 mM) + methyl succinate (10 mM) | 16.0 ± 1.0 (8)* | 7.6 ± 0.6 (8)* | 16.4 ± 0.6 (10)* | 12.5 ± 0.3 (8) |
| KIC (2 mM)                        | 3.9 ± 0.5 (7)* | 6.2 ± 0.1 (4)* | 19.6 ± 0.7 (8)* | 10.3 ± 0.4 (4) |
| MMS (10 mM)                       | 1.8 ± 0.4 (4)* | 7.8 ± 0.5 (4)* | 26.2 ± 2.2 (8)  | 19.3 ± 1.5 (6)* |
| KIC (10 mM)                       | 2.4 ± 0.8 (5)  | 8.3 ± 0.9 (4)* | 15.8 ± 1.4 (10)* | 7.9 ± 1.2 (8)* |

*p < 0.001 versus no secretagogue added.

TABLE 6
Combinations of insulin (non)secretagogues that increase insulin release also increase concentrations of acetoacetate and α-ketoglutarate in cultured rat pancreatic islets

Conditions were the same as described in Table 5. After 30 min, metabolism was stopped, and metabolites were assessed as described under “Experimental Procedures.” Relative insulin release data are from Table 1. Metabolite results are the mean ± S.E. with the number of replicate incubations in parentheses.

| Secretagogue added (concentration) | Relative insulin release | Metabolite concentration |
|------------------------------------|--------------------------|--------------------------|
|                                    | % control     | Control     | Acetoacetate | α-Ketoglutarate |
|                                    |               |             | nmol metabolite/mg islet protein | nmol metabolite/mg islet protein |
| None                               | 0.06 ± 0.03 (59) | 0.21 ± 0.07 (46) |              |              |
| KIC (2 mM)                         | 0.34 ± 0.02 (6)*  | 0.30 ± 0.02 (6)  |              |              |
| KIC (2 mM) + MMS (10 mM)           | 0.07 ± 0.08 (11) | 0.75 ± 0.09 (13)* |              |              |
| Pyruvate (5 mM)                    | 0.71 ± 0.09 (15)* | 1.2 ± 0.18 (15)*  |              |              |
| Pyruvate (5 mM) + MMS (10 mM)      | 0.07 ± 0.04 (13) | 0.76 ± 0.12 (17)* |              |              |
| Glucose (2 mM)                     | 0.55 ± 0.09 (10) | 1.3 ± 0.14 (21)  |              |              |
| Glucose (2 mM) + MMS (10 mM)       | 0.22 ± 0.10 (8)  | 0.22 ± 0.10 (12) |              |              |
| Glucose (16,7 mM)                  | 0.67 ± 0.17 (7)*  | 0.86 ± 0.17 (10)* |              |              |
| KIC (10 mM)                        | 0.30 ± 0.11 (14)* | 0.37 ± 0.14 (19) |              |              |
| KIC (10 mM) + methyl succinate (10 mM) | 1.3 ± 0.11 (18)* | 0.40 ± 0.06 (11)* |              |              |

*p < 0.001 versus no addition control or methyl succinate alone.

TABLE 7
Metabolite values in INS-1 cells incubated in the presence of a substimulatory concentration of a secretagogue and with or without methyl succinate

INS-1 cells 832/13 cells were maintained in RPMI 1640 medium (contains 11.1 mM glucose) plus 10% fetal bovine serum. Twenty-four hours before cells were harvested, the concentrations of glucose in the medium was lowered to 5 mM. Cells were harvested and suspended at a concentration of 3–4 mg/cell protein/ml in well oxygenated Krebs-Ringer bicarbonate solution containing 15 mM sodium Hepes buffer, pH 7.3, and 0.5% fetal bovine serum with gentle shaking at 37 °C. After 30 min, metabolism was stopped by adding five volumes of 6% perchloric acid. Protein was removed from the perchloric acid pellet by centrifugation, and it was neutralized with 30% KOH. Several experiments were performed with cells as monolayers on tissue culture plates instead of in suspension, and fold changes in metabolites were similar to those in cells maintained in suspension. Data from both types of experiments were averaged together with data for this table. Results are expressed as the mean ± S.E. with the number of replicate incubations in parentheses.

| Secretagogue added | Metabolite concentration |
|--------------------|--------------------------|
|                    | Malate       | Citrate   | Glutamate    | Aspartate   | Isocitrate |
|                    | nmol metabolite/mg cell protein |       |              |             |            |
| None               | 0.3 ± 0.1 (11) | 1.4 ± 0.2 (12) | 16.3 ± 2.8 (9) | 9.2 ± 2.2 (8) | 0.12 ± 0.09 (8) |
| KIC (2 mM)         | 0.9 ± 0.2 (12)* | 0.9 ± 0.2 (11) | 11.7 ± 2.4 (9) | 8.9 ± 2.4 (8) | 0.20 ± 0.08 (8) |
| MMS (10 mM)        | 1.8 ± 0.5 (11)* | 2.2 ± 0.5 (10) | 13.4 ± 1.4 (8) | 8.5 ± 2.0 (8) | 0.33 ± 0.11 (9) |
| KIC (2 mM) + MMS (10 mM) | 1.9 ± 0.3 (8)* | 2.9 ± 0.6 (10)* | 15.5 ± 2.1 (9) | 8.7 ± 2.1 (8) | 0.41 ± 0.12 (10) |
| Pyruvate (5 mM)    | 1.2 ± 0.2 (8)* | 2.3 ± 0.4 (9)* | 16.6 ± 2.9 (10) | 10.1 ± 1.3 (8) | 0.31 ± 0.04 (6) |
| Pyruvate (5 mM) + MMS (10 mM) | 2.3 ± 0.3 (12)* | 2.4 ± 0.3 (9)* | 14.3 ± 3.3 (8) | 9.6 ± 1.6 (8) | 0.37 ± 0.03 (6)* |
| Glucose (2 mM)     | 0.5 ± 0.1 (6)  | 1.5 ± 0.3 (9)  | 15.2 ± 2.8 (7) | 7.3 ± 0.8 (8) | 0.13 ± 0.06 (8) |
| Glucose (2 mM) + MMS (10 mM) | 1.3 ± 0.2 (8)* | 2.1 ± 0.3 (9)  | 16.0 ± 3.5 (8) | 10.7 ± 2.0 (8) | 0.31 ± 0.10 (8) |
| Glucose (11.1 mM)  | 1.00 ± 0.1 (4)* | 2.2 ± 0.4 (4)  | 15.1 ± 1.8 (4) | 7.0 ± 0.8 (4)  | 0.32 ± 0.07 (4)* |
| KIC (10 mM)        | 0.75 ± 0.2 (4)  | 2.1 ± 0.4 (4)  | 11.4 ± 2.4 (4) | 7.9 ± 0.9 (4)  | 0.23 ± 0.08 (4) |

*p < 0.02 versus no addition control.

*p < 0.01 versus no addition control.

*p < 0.001 versus no addition control.

*p < 0.05 versus no addition control.

such as methyl succinate alone or 10 mM KIC alone, did not stimulate insulin release from INS-1 832/13 cells (Table 7). As in islets, in INS-1 cells, the conditions that stimulated insulin release the most tended to cause the largest relative increases in acetoacetate and α-ketoglutarate, however with a few more exceptions (Table 8). As observed in islets, stimulatory glucose (11.1 mM) only moderately increased the levels of the two metabolites but caused a large stimulation of insulin release. Pyruvate, which is as potent an insulin stimulant as glucose in INS-1 832/13 cells (Table 3), caused only a moderate increase in acetoacetate and a large increase in α-ketoglutarate (Table 8). Also, 10 mM KIC alone, which does not stimulate insulin release
Synergistic Insulin Release by Nonsecretagogue Combinations

TABLE 8

| Secretagogue added (concentration) | Relative insulin release | Metabolite concentration | Acetocetate | α-Ketoglutarate |
|-----------------------------------|--------------------------|--------------------------|-------------|----------------|
| None                              | Control                  |                          | 0.0 ± 0.16 (12) | 0.53 ± 0.26 (12) |
| KIC (2 mM)                        | 1                        |                          | 0.76 ± 0.18 (12)*a 0.53 ± 0.12 (10) |
| MMS (10 mM)                      | 7                        |                          | 0.05 ± 0.21 (12) | 0.58 ± 0.16 (11) |
| KIC (2 mM) + MMS (10 mM)         | 13                       |                          | 1.4 ± 0.22 (11)b 1.5 ± 0.18 (11)b  |
| Pyruvate (5 mM)                  | 13                       |                          | 0.42 ± 0.07 (11)c 2.0 ± 0.18 (11)c  |
| Pyruvate (5 mM) + MMS (10 mM)    | 13                       |                          | 0.55 ± 0.24 (9)c 2.1 ± 0.33 (8)c  |
| Glucose (2 mM)                   | 13                       |                          | 0.14 ± 0.33 (11)c 0.44 ± 0.25 (10) |
| Glucose (2 mM) + MMS (10 mM)     | 13                       |                          | 0.44 ± 0.08 (10)c 0.84 ± 0.30 (10) |
| Glucose (11.1 mM)                | 12                       |                          | 0.44 ± 0.10 (6)c 0.78 ± 0.18 (4)c  |
| KIC (10 mM)                      | 1                        |                          | 1.0 ± 0.16 (6)b 1.5 ± 0.55 (4) |

*p < 0.01 versus no addition control or MMS alone.

*p < 0.001 versus no addition control or MMS alone.

*p < 0.05 versus no addition control or MMS alone.

in INS-1 832/13 cells, increased acetocetate and α-ketoacetate. Since acetocetate is in close proximity to several short chain acyl-CoAs in metabolic pathways (Fig. 1, reactions 28, 29, 30, and 31) and relative acetocetate levels in islets and INS-1 cells tended to correlate with insulin release, short chain acyl-CoAs were measured in secretagogue-stimulated cells.

Short Chain Acyl-CoA Measurements—INS-1 832/13 cells were treated with glucose alone, pyruvate alone, or 2 mM KIC plus 10 mM methyl succinate, which are conditions that stimulate insulin release in these cells, as well as with 1.5 mM glucose, KIC alone, and methyl succinate alone as controls. The levels of acetocetoy-CoA, acetoacetoy-CoA, succinoy-CoA, hydroxymethylglutaryl-CoA, and malonyl-CoA were measured with LC-MS/MS. Except for acetocetoy-CoA, which was increased about 1.5-fold, the levels of these acyl-CoAs were in general increased 2–3-fold by glucose and pyruvate and 2–5-fold by KIC plus methyl succinate (Table 9). KIC alone, at either a 2 or 10 mM concentration caused large increases in the levels of most of the acyl-CoAs, as did KIC plus methyl succinate. This indicates that, as might be expected from the metabolism of KIC to hydroxymethylglutaryl-CoA, acetyl-CoA, acetoacetate, acetocetoy-CoA, and malonyl-CoA (Fig. 1, reactions 24–31 and 13, 14, 15, and 23), provision of short chain acyl-CoAs could be part of the mechanism by which KIC complements methyl succinate to promote insulin release. However, since KIC alone does not stimulate insulin release in INS-1 cells, this indicates that other factors in addition to the synthesis of short chain acyl-CoAs are necessary for insulin secretion.

DISCUSSION

The synergistic insulin release by combinations of poor secretagogues or nonsecretagogues in pancreatic islets and INS-1 (832/13) cells, as well as the metabolic studies of these cells, support the ideas that net synthesis of citrate, anaplerosis, and mitochondrial synthesis of short chain acyl-CoAs are important for insulin secretion. These concepts are all interrelated.

Net Synthesis of Citrate—First, the synergistic insulin release results support the idea that net synthesis of citrate is important for insulin secretion (9, 10). Methyl succinate can directly supply intramitochondrial oxaloacetate (Fig. 1, reactions 8–10) but only indirectly supplies intramitochondrial acetyl-CoA via a circuitous pathway that involves export of succinate carbon to the cytosol as malate, its conversion to pyruvate by malic enzyme, reentry of carbon into mitochondria as pyruvate, and decarboxylation of the pyruvate catalyzed by pyruvate dehydrogenase (Fig. 1, reactions 8, 9, 12, and 1) (6). This pathway may be dampened in islets that have been cultured overnight, because methyl succinate alone cannot stimulate insulin release from these islets. However, each of the three compounds, KIC, glucose, and pyruvate, that permit methyl succinate to be an insulin secretagogue can provide mitochondrial acetyl-CoA. (The inability of methyl succinate to stimulate insulin release from the cultured islets is not due to the culture conditions lowering the activities of the enzymes that catalyze the formation of pyruvate, oxaloacetate, or acetyl-CoA from succinate (malic enzyme (Fig. 1, reaction 12), mitochondrial malate dehydrogenase (Fig. 1, reaction 10), or pyruvate dehydrogenase (Fig. 1, reaction 1)), because culture at 5 mM glucose in the media used does not lower the activities of these enzymes (19, 23). Glucose and pyruvate form acetyl-CoA via the pyruvate dehydrogenase reaction (Fig. 1, reaction 1). Mitochondrial acetyl-CoA and acetocetate are formed from KIC via the reactions catalyzed by the branched chain α-ketoacid dehydrogenase, isovaleryl-CoA dehydrogenase, 3-methylcrotonyl-CoA carboxylase, 3-methylglutaconyl hydratase, and hydroxymethylglutaryl-CoA lyase, respectively (Fig. 1, reactions 24–28). Mitochondrial acetocetate can be converted to acetocetoy-CoA via succinyl-CoA:3-ketoacid transferase (Fig. 1, reaction 30), and acetocetoy-CoA can be converted to more acetyl-CoA via the mitochondrial acetocetoy-CoA thiolase (Fig. 1, reaction 29). The oxaloacetate from methyl succinate can condense with the acetyl-CoA to form citrate in the mitochondria (Fig. 1, reaction 3). Citrate can be exported from the mitochondria to the cytosol, where it can be converted to precursors used for the synthesis of many compounds, such as short chain acyl-CoAs, which may have signaling roles. Also, citrate-derived acetyl-CoA can be converted to malonyl-CoA, which is used in lipid synthesis (Fig. 1, reactions 13 and 14) or to mevalonate (Fig. 1, reactions 15, 20, and 23).

3 M. J. MacDonald, unpublished data.
Anaplerosis—Second, the results support existing data that indicate that anaplerosis is important for insulin secretion. The fact that combinations of compounds that are synergistic for insulin release are also compounds that can provide both oxaloacetate and acetyl-CoA that can condense to increase the synthesis of citrate supports this idea. When citrate synthesis is increased, the synthesis of any citric acid cycle intermediate can be increased. The fact that 10 mM KIC by itself does not stimulate INS-1 cells to stimulate the synthesis of any citric acid cycle intermediate can be increased.

**TABLE 9**

Increases in various short chain acyl-CoAs in INS-1 832/13 cells stimulated by insulin secretagogues or combinations of (non)secretagogues. INS-1 cells were incubated in the presence of (non)secretagogues for 30 min, and short chain acyl-CoAs were measured as described under “Experimental Procedures.” Results are the mean ± S.E. with the number of replicate incubations in parentheses.

| Incubation condition | Acetyl-CoA | Acetoacetyl-CoA | Succinyl-CoA | Hydroxymethylglutaryl-CoA | Malonyl-CoA pmol acyl-CoA/mg cell protein |
|----------------------|------------|-----------------|-------------|--------------------------|------------------------------------------|
| Glucose (1.5 mM) (control) | 97 ± 6.6 (16) | 10 ± 6.6 (16) | 30 ± 2.0 (29) | 33 ± 2.9 (21) | 36 ± 3.1 (16) |
| Glucose (16.7 mM) | 149 ± 14 (11) | 23 ± 2.9 (11) | 58 ± 4.8 (20) | 52 ± 5.5 (20) | 120 ± 19 (5) |
| Pyruvate (5 mM) | 146 ± 15 (12) | 22 ± 4.3 (12) | 58 ± 5.5 (21) | 58 ± 5.8 (16) | 119 ± 18 (7) |
| KIC (2 mM) | 139 ± 9.3 (26) | 28 ± 2.8 (16) | 139 ± 13 (16) | 60 ± 13 (9) | 132 ± 12 (9) |
| KIC (2 mM) + MMS (10 mM) | 118 ± 12 (8) | 36 ± 4.1 (13) | 143 ± 18 (5) | 60 ± 9 (5) | 134 ± 26 (7) |
| KIC (10 mM) | 162 ± 34 (6) | 31 ± 3.9 (7) | 150 ± 23 (11) | 54 ± 11 (4) | 170 ± 55 (5) |
| MMS (10 mM) | 129 ± 15 (10) | 16 ± 3.0 (10) | 75 ± 15 (9) | 28 ± 1.4 (5) | 61 ± 13 (7) |

* p ≤ 0.05 versus 1.5 mM glucose alone.

* p ≤ 0.001 versus 1.5 mM glucose alone.

* p ≤ 0.01 versus 1.5 mM glucose alone.
ulate insulin release from INS-1 cells (Table 3) indicates that more than ATP synthesis is necessary for insulin secretion. KIC by itself can increase acetyl-CoA (see Table 9) (via reactions 24–30 shown in Fig. 1), which can be metabolized to CO₂ in the citric acid cycle to produce 95% of the ATP any cell needs. If only ATP were necessary for insulin secretion, KIC by itself could stimulate insulin release in INS-1 cells. Methyl succinate, which can supply oxaloacetate to condense with acetyl-CoA to form citrate (Fig. 1, reaction 3), enables KIC to be a strong secretagogue (and vice versa).

These observations are consistent with previous data that indicate that the rate of carboxylation of glucose-derived pyruvate to oxaloacetate correlates more closely with the glucose concentration islets are exposed to (and thus with insulin release) than does the rate of decarboxylation of glucose-derived pyruvate to acetyl-CoA and its metabolism to CO₂ in the citric acid cycle (3, 4, 6). This idea is also supported by studies of Lu et al. (8), who used NMR to estimate pyruvate cycling in INS-1 832/13 cells. They observed that the rate of pyruvate cycling directly correlated with the ability of various INS-1 cell lines to release insulin in response to glucose. Cline et al. (13), using NMR and 13C-isotopomer analysis in studies of INS-1 832/13 cells, also showed that flux through pyruvate carboxylase correlated with insulin secretion.

Short Chain Acyl-CoAs—Third, the results support the hypothesis that short chain acyl-CoAs are important for insulin secretion (2, 14). In general, acetoacetate and α-ketoglutarate were increased by conditions that stimulated insulin release in both islets (Table 6) and INS-1 832/13 cells (Table 8). α-Ketoglutarate, via the α-ketoglutarate dehydrogenase reaction, is a direct precursor of mitochondrial succinyl-CoA (Fig. 1, reaction 6). Succinyl-CoA can increase mitochondrial acetoacetyl-CoA via the succinyl-CoA:3-ketoacyl-CoA transferase reaction (Fig. 1, reaction 30). Acetoacetyl-CoA equivalents can then be converted to acetyl-CoA, which is exported from the mitochondria to the cytosol as citrate and converted back to acetyl-CoA in the ATP citrate lyase reaction (Fig. 1, reaction 13) as described above. The acetyl-CoA can be converted to acetoacetyl-CoA via the cytosolic acetoacetyl-CoA thiolase (Fig. 1, reaction 23). In addition, acetoacetate can be exported from the mitochondria to the cytosol and converted to acetoacetyl-CoA via the acetoacetyl-CoA synthetase reaction (Fig. 1, reaction 31). Acetoacetate lies in close proximity to several short chain acyl-CoAs in metabolic pathways. Therefore, when acetoacetate is increased in any cell type, it almost always indicates that acetoacetyl-CoA and hydroxymethylglutaryl-CoA are also increased (37) (Fig. 1, reactions 28, 29, 30, 31, 23, and 15). Levels of acetyl-CoA, succinyl-CoA, acetoacetyl-CoA, hydroxymethylglutaryl-CoA, and malonyl-CoA have been reported in glucose-stimulated clonal insulin cell lines and islets measured with HPLC (9, 38, 39). Table 9 shows concentrations of short chain acyl-CoAs measured by LC-MS/MS, which more specifically identifies these metabolites. These measurements indicate that synergistic combinations of metabolizable nonsecretagogues used in the current work, as well as the insulin stimulants glucose alone and pyruvate alone, generally caused 1.5–5-fold increases in these short chain CoAs in INS-1 832/13 cells. Except for increasing succinyl-CoA, methyl succinate alone did not increase any of the acyl-CoAs measured. KIC alone, as expected from the routes of its metabolism (Fig. 1, reactions 24–31 and 13, 14, 15, and 23 as well as reactions 19 plus 6) increased each of the acyl-CoAs measured, suggesting that providing short chain acyl-CoAs might be part of the mechanism by which it synergizes with methyl succinate to stimulate insulin release. However, since KIC alone does not stimulate insulin release from INS-1 832/13 cells, more than these acyl-CoAs are required for insulin secretion. For example, methyl succinate alone can supply cytosolic NADPH equivalents (via malic enzyme (Fig. 1, reaction 12). In combination with a source of acetyl-CoA, it can also supply NADPH via cytosolic isocitrate dehydrogenase (Fig. 1, reaction 33), as discussed in Refs. 2, 6, and 8. NADPH is required for fatty acid elongation and lipid synthesis, and this may be one of the reasons why KIC and methyl succinate synergize to permit insulin release. NADPH would permit malonyl-CoA to be used for lipid synthesis.

Insulin Release by Pyruvate in INS-1 Cells—Pyruvate’s causing insulin release from INS-1 832/13 cells is somewhat novel and deserves a brief comment. The reason that pyruvate stimulates insulin release from INS-1 cells and not from pancreatic islets (24–28) is most likely because INS-1 cells possess a high level of a monocarboxylate transporter (27, 35), enabling pyruvate to be rapidly taken up and used for anaplerosis via its carboxylation to oxaloacetate by pyruvate carboxylase and also decarboxylated to acetyl-CoA by pyruvate dehydrogenase and used for synthesis of citric acid, which can be metabolized in the citric acid cycle to produce energy or exported from the mitochondria to the cytosol for other uses. Pyruvate’s metabolism in INS-1 cells contrasts with glucose metabolism, which is much slower, because glucokinase modulates the flux of glucose carbon into the glycolytic pathway, where it is slowly converted to pyruvate.

CONCLUSIONS

The results of insulin release experiments and the measurements of metabolites combined with knowledge of well established metabolic pathways suggest a new mechanism by which anaplerosis signals or supports insulin secretion. This is in part by causing increases in certain short chain acyl-CoAs (Table 9). The three agents, KIC, glucose (2 mm), or pyruvate, that synergize with methyl succinate to stimulate insulin release in rat or human islets supply acetyl-CoA (Fig. 1, reactions 24–28 for KIC or reaction 1 for glucose and pyruvate), which can condense with oxaloacetate from methyl succinate (Fig. 1, reactions 21, 8, 9, and 10) to form citrate (Fig. 1, reaction 3). Citrate can export equivalents of short chain acyl-CoAs from mitochondria to the cytosol (Fig. 1, reaction 13). The export of citrate, isocitrate, and malate from mitochondria to the cytosol can export NADPH equivalents to the cytosol (Fig. 1, reactions 13, 11, and 12 for citrate, reaction 33 for isocitrate, and reaction 12 for malate). There is evidence for a role for NADPH in insulin secretion (2, 6, 8, 13, 14). The pathways by which KIC is metabolized suggest why it is the most potent of the three synergizers in permitting methyl succinate to be an insulin secretagogue. KIC can directly increase the levels of several mitochondrial and cytosolic short chain acyl-CoAs as well as acetoacetate, which is a molecule...
that can also export CoA equivalents from the mitochondria to the cytosol (Fig. 1, reactions 24–31 plus 23, 14, and 15). Glucose and pyruvate can directly increase acetyl-CoA, which can be converted to other mitochondrial and cytosolic short chain acyl-CoAs via reactions 1, 3, 13, 14, 15, and 23 shown in Fig. 1.

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