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Recognition of Insulin-Releasing Fuels by Pancreatic B-Cells

α-Ketoisocaproic Acid is an Appropriate Model Compound to Study the Role of B-Cell Metabolism

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SUMMARY

PANTEN, U., J. BIERMANN, AND W. GRAEN: Recognition of insulin-releasing fuels by pancreatic B-cells: α-ketoisocaproic acid is an appropriate model compound to study the role of B-cell metabolism. *Mol. Pharmacol.* **20**:76–82 (1981).

α-Ketoisocaproic acid released insulin from isolated mouse islets with a threshold concentration at 2–3 mM and a maximal effect at 15–20 mM. Stimulation of insulin secretion was accompanied by small increases of cyclic AMP accumulation by islets which could be prevented by omission of Ca^{2+} from the incubation media. Extramitochondrial metabolites that could arise from α-ketoisocaproic acid released much less insulin than their mother substance. Accumulation of intramitochondrial CoA compounds typical for degradation of α-ketoisocaproic acid probably did not cause the specific B-cell response to this keto acid. It is concluded that metabolites do not represent primary signals during α-ketoisocaproic acid-induced insulin secretion. The experimental data are compatible with the view that increase of intramitochondrial production of reducing equivalents is necessary for recognizing insulin-releasing fuels by B-cells.

INTRODUCTION

Glucose is the main physiological stimulator of insulin secretion, but it is unknown whether the glucose molecule is recognized by specific membrane receptors or by glucose metabolism in the B-cell that generates a change in the concentration of intracellular trigger metabolites (1, 2). The widely branched glucose metabolism yields many intermediates which are candidates for such a role. These metabolites largely do not penetrate plasma membranes. Therefore, conclusive experiments cannot be performed by exposing pancreatic islets to these compounds and measuring insulin release.

From correlation between islet content of NAD(P)H,[1] Ca^{2+} handling and insulin secretion under a variety of nutritional conditions, Malaisse and co workers (3) reasoned that changes in NAD(P)H concentration account for the insulin-releasing effect of fuels. Measurement of whole-tissue contents of NAD(P)H, however, does not allow definite conclusions on the concentrations of free NAD(P)H which differ by several orders of magnitude in the various cellular compartments (4). Moreover, recordings of NAD(P)H-fluorescence of intact islets demonstrated that, in islet cells, NAD(P)H pools exist which dissociate from insulin release (5, 6).

In the present study, we have tried a different approach to reveal metabolic events in B-cells which may mediate fuel-induced insulin secretion. We chose α-ketoisocaproic acid as model compound supplementing previous fragmentary investigations on its mode of insulin-releasing action (5, 7–9). This first metabolite of leucine stimulates a prompt, sustained, and strong insulin release from isolated pancreatic islets in the absence of other fuels or secretagogues (5) and its metabolism is less complicated than glucose metabolism. This gives a chance to rule out that metabolites arising from α-ketoisocaproic acid are trigger intermediates and to leave enhanced intramitochondrial production of reducing equivalents as metabolic releasing signal (Fig. 1).

Generalization of findings on α-ketoisocaproic acid recognition requires similarities in later steps of α-ketoisocaproic acid- and glucose-induced insulin release. Such similarities have been documented regarding several putative coupling factors (10), but measurements of cyclic AMP suggested a different mode of action of α-ketoisocaproic acid and glucose in stimulating insulin release (11). Although those findings may have been overinterpreted, we performed a more detailed investigation on

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[1] The abbreviations used are: NAD(P)H, reduced pyridine nucleotides; ob/ob, obese-hyperglycemic.

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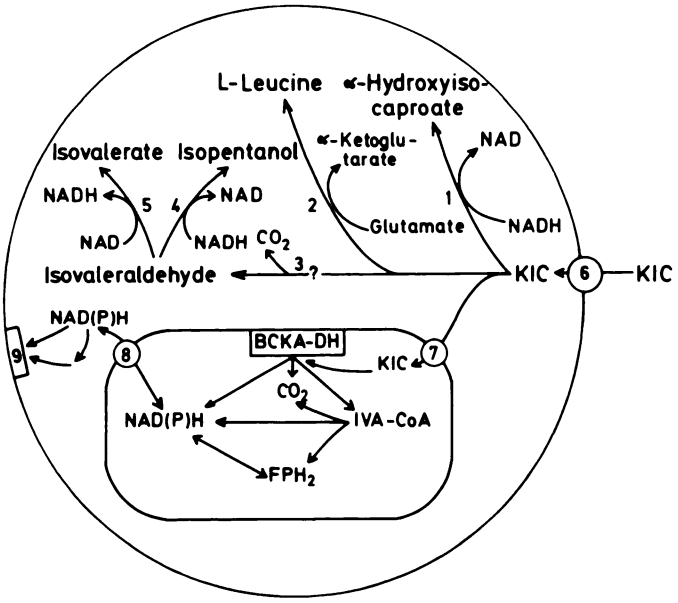

FIG. 1. *Transport and metabolism of α-ketoisocaproic acid (KIC)* *1*, Lactate dehydrogenase; *2*, transaminase; *3*, KIC-decarboxylase; *4*, aldehyde reductase; *5*, aldehyde dehydrogenase; *6*, anion carriers in the plasma membrane; *7*, anion carriers in the inner mitochondrial membrane; *8*, hydrogen transport mechanisms; *9*, hypothetical membrane receptor; *BCKA-DH*, branched-chain keto acid dehydrogenase; *IVA-CoA*, isovaleryl CoA; FPH_2, reduced flavoproteins. Reduction of KIC or oxidation of isovaleraldehyde in the intramitochondrial space is not shown.

α-ketoisocaproic acid-induced cyclic AMP accumulation by pancreatic islets.

MATERIALS AND METHODS

Chemicals. The following substances were used: collagenase (Type IV) from Worthington Biochemical Corporation, Freehold, N. J.; bovine serum albumin (Fraction V), sodium succinate, and L-leucine (p.a.) from Serva, Heidelberg, Germany; isopentanol (puriss.), isovaleraldehyde, isovaleric acid (puriss.), and 3,5-diaminobenzoic acid hydrochloride from Fluka, Buchs, Switzerland; sodium α-ketoisocaproate, sodium α-ketoisovalerate, L-α-hydroxyisocaproic acid, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, calf thymus DNA (Type I), cyclic AMP, and charcoal (hydrochloric acid-washed) from Sigma Chemical Company, St. Louis, Mo.; crystalline mouse insulin from Novo, Bagsvaerd, Denmark; Aqualuma from Lumac, Meise, Belgium; cyclic AMP radioimmunoassay kit from Becton, Dickinson, Orangeburg, N. Y.; ^{125}I-labeled bovine insulin from Behringwerke, Frankfurt, Germany; L-[1-^{14}C]leucine from Amersham Buchler, Braunschweig, Germany; L-[2-^{14}C]leucine from CEA Sorin, Gif-Sur-Yvette, France. ^{14}C-Labeled α-ketoisocaproic acid was prepared and analyzed as described (12). All other reagents were analytical grade from Merck, Darmstadt, Germany.

Media. Basal medium was a modified Krebs-Ringer bicarbonate buffer (143 mM Na^+, 5.9 mM K^+, 2.5 mM Ca^{2+}, 1.2 mM Mg^{2+}, 128 mM Cl^-, 1.2 mM SO_4^{2-}, 1.2 mM PO_4, 20.2 mM HCO_3^-, 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, 0.2% albumin, and OH^- to give pH 7.4 after equilibration with O_2 + CO_2 (95:5, v/v) at 37°). In some experiments calcium was omitted from the media and 1 mM ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid (NaOH to give pH 7.4) was added. Test substances for perifusion or incubation experiments were dissolved in basal medium. No corrections for the sodium content of test substances were made. Stock solutions (1 M) of L-α-hydroxyisocaproate or isovalerate were prepared daily by neutralization with NaOH. The solubility of isovaleraldehyde in water is low. A medium saturated with isovaleraldehyde was prepared by adding 5 μl of isovaleraldehyde to 5 ml of basal medium and shaking at 20° for 5 min. A medium with 50% saturation was made by replacing half of the distilled water for medium preparation by water saturated with isovaleraldehyde.

Isolation of pancreatic islets. Ob/ob mice (noninbred, 28–32 weeks old, 50–60 g, deprived of food for 22–25 hr) of either sex or male albino mice (NMRI, 11–15 weeks old, 35–45 g, fed ad libitum) were used. Pancreatic islets were isolated by collagenase digestion (13) in basal medium supplemented with 4 mM D-glucose.

Perifusion of pancreatic islets. Perifusions at 0.9 ml/min at 37° were performed as described (14). Insulin was determined as described below.

Incubation of pancreatic islets. Batches of 40 albino mouse islets or 20 medium-sized ob/ob mouse islets were incubated at 37° in 40 μl of basal medium (without any substrate) contained in small polypropylene tubes (tubes 30/8, Sarstedt, Nümbrecht, Germany). The tubes were placed in miniscintillation flasks (Lumac, Meise, Belgium) which contained 0.9 ml of water. The flasks were then gassed for 1 min with O_2 + CO_2 (95:5, v/v) and closed. After 45 min, 30 μl of medium were removed and replaced by 30 μl of basal medium without additions or with the test substances. A one-minute gassing and a 5-, 20-, or 60-min incubation period followed.

For determination of insulin release, 20 μl (unless stated otherwise under Results) of medium were removed after the incubation period and immunoassayed with mouse insulin as reference, using ammonium sulfate to precipitate the antibody-bound insulin (15). After dilution of the samples, none of the tested compounds interfered with the immunoassay.

When $^{14}CO_2$ production was to be measured, 100 μl of 1 M NaOH were injected into the outer flask, 40 μl of citrate buffer (0.865 M Na^+, 0.425 M citrate, pH 4.9) into the inner tube, and the flask was placed in an ice bath. The latter manipulations were performed within 30 sec. After 3 hr at 0°, 77.5% of the $^{14}CO_2$ had been trapped by NaOH. The radioactivity was measured after addition of 5.5 ml of Aqualuma. For each test incubation, a blank value was obtained by incubating the medium without islets. Corrections for incomplete recovery were made.

Incubations for analysis of total cyclic AMP were stopped by pipetting 80 μl of cold 7.5% trichloroacetic acid into the inner tube. Cyclic AMP in the medium was measured by transferring 30 μl of medium without islets to a tube containing 80 μl of 7.5% trichloroacetic acid and 10 μl of basal medium. Immediately after acid addition, the stoppered tube was sonicated in an ice bath by

pressing it against the tip of a Branson sonifier (Type S125, 5 × 3 sec at position 8). After centrifugation (2 min at 10,000 × *g*), 100 μl of the supernatant were added to 10 μl 1 N HCl and the 20 μl of residue was used for DNA measurement (after neutralization with 5 μl of NaOH). The supernatant was washed three times with 1 ml of ethyl ether saturated with water. The solution was dried at 56° with a constant stream of nitrogen. The residue was dissolved in 200 μl of sodium acetate buffer (0.05 M; pH 6.2) and stored at −30° until analyzed by using the cyclic AMP kit of Becton, Dickinson. The different media did not interfere with the radioimmunoassay. Recovery was 97–106% as determined by preincubating and incubating 5 × 10^{-14} to 10^{-11} mole of cyclic AMP in 40 μl of basal medium at 37°.

Determination of islet DNA content. DNA content of the islets was measured by a modification (12) of the method of Kissane and Robins (16). DNA standards in adequate volumes of medium or mixtures of medium and quenching solutions were run together with the samples. With the exception of isovaleraldehyde, test substances did not interfere with the DNA determinations. The DNA content of mouse islets was 3.8% of the islet dry weight.

Presentation of results. Assuming homogeneous distribution of insulin in the incubation medium, insulin release was calculated from the amounts measured in aliquots. Results are presented as means and standard errors. Significances were calculated by two-tailed nonparametric tests. A p value below the 5% level was considered significant. Wilcoxon's matched-pairs signed rank test was used if only one paired comparison was made per experimental series. Multiple comparisons of related samples were performed by Friedman's test, subsequent use of the Friedman technique for the comparison of interesting pairs, and estimation of significance by a sequential Bonferroni procedure (17). When noted, the *U*-test of Wilcoxon and of Mann and Whitney was used.

RESULTS

Effects of extracellular keto acid concentrations on insulin secretion. Figure 2 shows that 5, 10, or 20 mM α-ketoisocaproic acid stimulated a sustained insulin release from perifused mouse islets. As compared with the last 4-min control fraction, the insulin secretion during the first 4-min test period was always significantly higher ($p \leq 0.01$). During the 32-min test period, the response to 20 mM α-ketoisocaproic acid was slightly higher than to 10 mM. A small, significant ($p < 0.01$) insulin release was triggered by α-ketoisovaleric acid only during the first 8 min after exposure to a 20 mM concentration (Fig. 2).

Similar concentration-release relationships were seen with mouse islets incubated for 60 min in 40 μl of medium (Fig. 3). The threshold concentration of α-ketoisocaproic acid necessary to stimulate insulin secretion in the microsystem which was used for metabolic studies was 2–3 mM. As compared with the corresponding controls without additions, insulin release in the presence of 3 mM α-ketoisocaproic acid was significantly higher ($p < 0.05$). No significant differences between secretion triggered by 10, 15, or 20 mM α-ketoisocaproic acid were observed (*U*-test). High concentrations of α-ketoisovaleric acid induced only minute increases of insulin secretion ($p < 0.02$ at 15 mM; $p < 0.05$ at 20 mM).

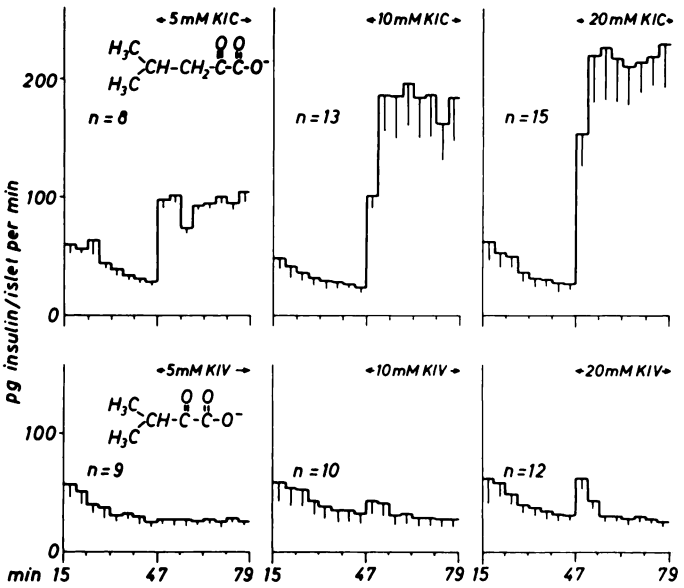

FIG. 2. *Effect of α-ketoisocaproic acid (KIC) or α-ketoisovaleric acid (KIV) on insulin release by perifused pancreatic islets*

Groups of 90 albino mouse islets were perifused for 47 min with basal medium and then for 32 min with basal medium containing the indicated concentrations of keto acids. The effluent was collected at 4-min intervals. Results are presented as mean values ± standard error of *n* experiments.

With a maximally effective concentration of α-ketoisocaproic acid, a small stimulation of insulin release could be demonstrated in the microincubation system within 5 min of exposure to the keto acid (Table 7). This response was partially hidden by insulin which was secreted during the preincubation period and remained in the incubation tubes.

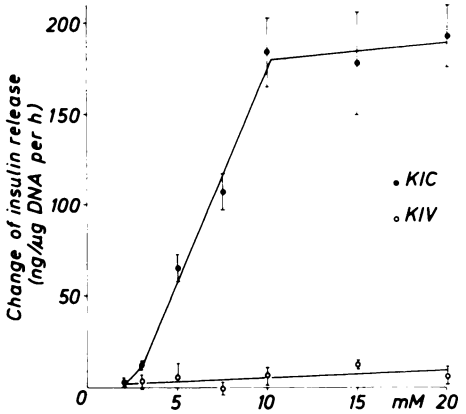

FIG. 3. *Effect of α-ketoisocaproic acid (KIC) or α-ketoisovaleric acid (KIV) on insulin release by incubated pancreatic islets*

Groups of 40 albino mouse islets were incubated for 45 min in basal medium and then for 60 min in basal medium or basal medium containing the indicated concentrations of keto acids. Change of insulin release was calculated by subtracting the corresponding basal release. The mean basal secretion ranged between 16.2 ± 1.6 and 23.8 ± 2.3 ng of insulin per microgram of DNA per hr. Results are presented as mean values ± standard error of 12–20 observations with KIC and of 8–14 observations with KIV.

Effects of metabolites of α-ketoisocaproic acid on insulin secretion. Figure 1 shows that in the extramitochondrial space several metabolites can arise from α-ketoisocaproic acid which are potential triggers of insulin release. L-α-Hydroxyisocaproic acid (10 mM) caused only a very small stimulation of insulin secretion in the absence of other fuels (Table 1). In order to rule out that lack of insulin-releasing effects was due to insufficient energy supply or other inhibition mechanisms, metabolites were also tested in the presence of a low α-ketoisocaproic acid concentration. In the presence of α-ketoisocaproic acid, L-α-hydroxyisocaproic acid did not change insulin release (Table 1). L-Leucine (20 mM) was much less potent than α-ketoisocaproic acid (Table 1).

When medium containing 5 mM α-ketoisocaproic acid was saturated with isovaleraldehyde, insulin secretion (nanograms per 60 min per 40 islets) decreased significantly ($p < 0.005$) from a control value of 84.3 ± 8.0 ($n = 10$) to 56.9 ± 4.7 ($n = 10$). When medium containing 5 mM α-ketoisocaproic acid was saturated to 50% with isovaleraldehyde, no significant change in insulin secretion was observed (72.9 ± 6.3 or 69.4 ± 7.2, respectively; $n = 10$). In the absence of α-ketoisocaproic acid, too, 50% saturation with isovaleraldehyde did not change insulin secretion significantly (results not shown).

High concentrations of isopentanol or isovaleric acid inhibited α-ketoisocaproic acid-induced insulin release (Tables 2 and 3). At noninhibitory concentrations, isopentanol (0.8 mM) or isovaleric acid (2 mM) did not stimulate insulin release in the absence or in the presence of α-ketoisocaproic acid.

Effects of α-ketoisovaleric acid or succinate on α-ketoisocaproic acid-induced insulin release and degradation of α-ketoisocaproic acid by islets. To elucidate the importance of intramitochondrial CoA esters (Fig. 1) for recognition of α-ketoisocaproic acid as an insulin-releasing signal, the effects of the mitochondrial substrates α-ketoisovaleric acid and succinate on islet function were tested. In contrast to succinate, α-ketoisovaleric acid (20 mM) enhanced α-ketoisocaproic acid-induced insulin release from albino or ob/ob mouse islets significantly (Table 4). With the same incubation conditions, however, α-ketoisovaleric acid (20 mM) decreased the oxidative decarboxylation of [1-^{14}C]α-ketoisocaproic acid by 26% with albino mouse islets and by 47% with ob/ob mouse islets (Table 5). In parallel, CO_2 production from [2-^{14}C]α-ketoisocaproic acid was inhibited by 22% with albino mouse islets and by 34% with ob/ob mouse islets. Succinate had no significant effect on degradation of α-ketoisocaproic acid by ob/ob mouse islets (Table 5).

Effects of keto acids on cyclic AMP content of pancreatic islets and their incubation media. α-Ketoisocaproic acid (15 mM), which triggered a maximal insulin release, enhanced the total cyclic AMP content in islets and their incubation media by about 100% (Table 6, C-D). This increase, however, was prevented in Ca^{2+}-free media (Table 6, A-B). About one-third of the total cyclic AMP was released into the media (Table 6, E-F) and this release was enhanced significantly by α-ketoisocaproic acid (Table 6, E-F).

Within 5 min, α-ketoisocaproic acid (15 mM) had induced a 62% elevation of the total cyclic AMP in islets

TABLE 1

Effects of L-α-hydroxyisocaproic acid or L-leucine on insulin release

Albino mouse islets were incubated for 45 min without substrates and then incubated for 60 min in media with the additions shown. Each experimental series consisted of simultaneous incubations in four different media. Results are presented as mean values ± standard error. The number of experiments is shown in parentheses. Significance of differences between the treatments was calculated by using Friedman's technique. KIC, α-ketoisocaproic acid.

Additions	Insulin release[a]
	ng/μg DNA/hr
Experimental series I	
A: none	19.1 ± 1.8 (10)
B: KIC (5 mM)	91.2 ± 10.9 (10)
C: hydroxyisocaproate (10 mM)	27.6 ± 3.2 (10)
D: KIC (5 mM) + hydroxyisocaproate (10 mM)	89.7 ± 10.3 (10)
Experimental series II	
E: none	20.4 ± 2.0 (10)
F: KIC (5 mM)	71.0 ± 7.8 (10)
G: leucine (20 mM)	35.9 ± 3.9 (10)
H: KIC (5 mM) + leucine (20 mM)	100.2 ± 19.1 (10)

[a] Significance: A-B, $p < 0.01$; B-C, $p < 0.01$; B-D, not significant, (NS); A-C, $p < 0.05$; E-F, $p < 0.01$; F-G, $p < 0.01$; F-H, NS; E-G, $p < 0.01$.

TABLE 2

Effects of isopentanol on insulin release

See Table 1 for further details. KIC, α-ketoisocaproic acid.

Additions	Insulin release[a]
	ng/μg DNA/hr
Experimental series I	
A: none	19.7 ± 2.3 (10)
B: KIC (5 mM)	73.1 ± 11.1 (10)
C: KIC (5 mM) + isopentanol (10 mM)	25.4 ± 3.5 (10)
D: KIC (5 mM) + isopentanol (2 mM)	61.6 ± 5.4 (10)
Experimental series II	
E: none	23.6 ± 2.0 (10)
F: KIC (5 mM)	73.8 ± 12.5 (10)
G: KIC (5 mM) + isopentanol (0.8 mM)	85.2 ± 11.9 (10)
H: isopentanol (0.8 mM)	26.6 ± 4.2 (10)

[a] Significance: A-B, $p < 0.01$; B-C, $p < 0.05$; B-D, not significant (NS); E-F, $p < 0.01$; F-G, NS; E-H, NS.

TABLE 3

Effects of isovaleric acid on insulin release

See Table 1 for further details. KIC, α-ketoisocaproic acid.

Additions	Insulin release[a]
	ng/μg DNA/hr
Experimental series I	
A: none	20.0 ± 2.0 (10)
B: KIC (5 mM)	56.1 ± 9.8 (10)
C: KIC (5 mM) + isovalerate (10 mM)	22.0 ± 3.0 (10)
D: KIC (5 mM) + isovalerate (5 mM)	48.5 ± 9.9 (10)
Experimental series II	
E: none	20.8 ± 2.1 (10)
F: KIC (5 mM)	61.7 + 4.5 (10)
G: KIC (5 mM) + isovalerate (2 mM)	59.7 + 5.6 (10)
H: isovalerate (2 mM)	22.1 + 2.2 (10)

[a] Significance: A-B, $p < 0.01$; B-C, $p < 0.01$; B-D, not significant (NS); E-F, $p < 0.01$; F-G, NS; E-H, NS.

TABLE 4

Effects of α-ketoisovaleric acid (KIV) or succinate on insulin release induced by α-ketoisocaproic acid (KIC)

Albino mouse or ob/ob mouse islets were used. For further details see Table 1.

Additions	Insulin release[a]
	ng/μg DNA/hr
Albino mouse series	
A: none	20.6 ± 4.4 (10)
B: KIC (5 mM)	65.6 ± 8.4 (10)
C: KIC (5 mM) + succinate (20 mM)	83.9 ± 14.6 (10)
D: KIC (5 mM) + KIV (20 mM)	133.5 ± 9.8 (10)
ob/ob Mouse series[b]	
E: none	34.3 ± 5.3 (10)
F: KIC (5 mM)	113.7 ± 15.0 (10)
G: KIC (5 mM) + succinate (20 mM)	135.3 ± 20.6 (10)
H: KIC (5 mM) + KIV (20 mM)	208.4 ± 28.7 (10)

[a] Significance. A-B, $p < 0.01$; B-C, not significant (NS); B-D, $p < 0.01$; E-F, $p < 0.01$; F-G, NS; F-H, $p < 0.01$.

[b] Insulin release was measured in 30-μl aliquots.

and media (Table 7). The further increase of the total cyclic AMP content with prolonged incubation periods may indicate continuous cyclic AMP release into the media (Table 7). A cyclic AMP response was not induced by the nonsecretagogue α-ketoisovaleric acid (Table 7).

The relationships between α-ketoisocaproic acid concentrations and insulin secretion on the one hand and total cyclic AMP content on the other hand were not identical, but apparently covered the same concentration ranges. Both responses of pancreatic islets were maximal at 15–20 mM α-ketoisocaproic acid (Fig. 3, Table 7). 5 mM α-ketoisocaproic acid caused 35% of the maximal insulin-releasing effect and 47% of the maximal cyclic AMP increase (Table 7).

DISCUSSION

Because of the very limited amounts of islet tissue available (usually a few micrograms of DNA per incubation series), metabolic studies are performed in microincubation systems. However, pancreatic islets are then exposed to rather high concentrations of their own secretory products. Insulin, glucagon, somatostatin, and pancreatic polypeptide modulate B-cell function and metabolism.

In our perifusion system, the effects of secretory prod-

TABLE 5

Degradation of ^{14}C-labeled α-ketoisocaproic acid (KIC) by pancreatic islets

Islets were incubated for 45 min without substrates and then incubated for 60 min in media with the additions shown. Each experimental series consisted of paired incubations in media with [1-^{14}C]KIC (1 Ci/mole) or [2-^{14}C]KIC (1 Ci/mole). Results are presented as mean values ± standard error. Number of experiments is shown in parentheses. KIV, α-ketoisovaleric acid.

Additions	CO_2-production[a]	
	[1-^{14}C]	[2-^{14}C]
	nmoles/μg DNA/per hr	
Albino mouse series		
KIC (5 mM)	2.07 ± 0.07 (6)	1.26 ± 0.05 (7)
KIC (5 mM) + KIV (20 mM)	1.54 ± 0.06 (6)*	0.98 ± 0.10 (7)*
ob/ob Mouse series I		
KIC (5 mM)	1.12 ± 0.10 (8)	0.73 ± 0.09 (8)
KIC (5 mM) + KIV (20 mM)	0.59 ± 0.08 (8)**	0.48 ± 0.09 (8)**
ob/ob mouse series II		
KIC (5 mM)	1.27 ± 0.15 (8)	0.71 ± 0.13 (8)
KIC (5 mM) + succinate (20 mM)	1.04 ± 0.17 (8)	0.63 ± 0.11 (8)

[a] Significance of difference between values in the absence or presence of α-ketoisovaleric acid or succinate by using Wilcoxon's paired test: * $p < 0.05$; ** $p \leq 0.01$.

TABLE 6

Effects of Ca^{2+} on cyclic AMP content of media and pancreatic islets

Albino mouse islets were incubated for 45 min without substrates and then incubated for 20 min with or without Ca^{2+}. See Table 1 for further details. +, With addition; −, without addition. KIC, α-ketoisocaproic acid.

Group	Ca^{2+}	KIC (15 mM)	Cyclic AMP[a]
			pmoles/μg DNA
Total content series			
A	−	−	0.21 ± 0.04 (12)
B	−	+	0.22 ± 0.05 (12)
C	+	−	0.18 ± 0.04 (12)
D	+	+	0.37 ± 0.09 (12)
Medium content series			
E	+	−	0.07 ± 0.04 (16)
F	+	+	0.11 ± 0.04 (16)

[a] A-B, not significant (NS); A-C, NS; B-D, $p < 0.01$; C-D, $p < 0.01$; E-F, $p < 0.01$.

TABLE 7

Effects of incubation time and different concentrations of α-ketoisocaproic acid (KIC) on insulin release and total cyclic AMP content of pancreatic islets + medium

Albino mouse islets were incubated for 45 min without substrates and then incubated for 5, 20, or 60 min in media with the additions shown. Each experimental series consisted of simultaneous incubations in three different media. Results are presented as mean values ± standard error. Number of experiments is shown in parentheses. KIV, α-ketoisovaleric acid.

Additions	Insulin release[a]	Cyclic AMP[a]
	ng/μg DNA/incubation period	*pmoles/μg DNA*
5-min incubation series[b]		
none	13.7 ± 1.0 (8)	0.21 ± 0.05 (12)
KIC (15 mM)	20.4 ± 2.2 (8)*	0.34 ± 0.08 (12)*
KIV (15 mM)	13.5 ± 1.6 (8)**	0.21 ± 0.04 (12)**
20-min incubation series[b]		
none	17.1 ± 3.2 (8)	0.21 ± 0.06 (12)
KIC (15 mM)	69.1 ± 12.1 (8)*	0.37 ± 0.09 (12)*
KIV (15 mM)	21.9 ± 2.4 (8)**	0.19 ± 0.06 (12)**
60-min incubation series		
none	18.3 ± 3.4 (8)	0.25 ± 0.08 (8)
KIC (15 mM)	164.8 ± 20.5 (8)*	0.47 ± 0.13 (8)*
KIV (15 mM)	25.3 ± 3.9 (8)**	0.26 ± 0.10 (8)**
20-min incubation series		
none	18.2 ± 3.6 (10)	0.26 ± 0.07 (11)
KIC (5 mM)	39.7 ± 8.4 (10)*	0.34 ± 0.05 (11)*
KIC (20 mM)	80.2 ± 9.9 (10)*	0.43 ± 0.06 (11)*

[a] Significance of difference from corresponding controls (without additions) by using Friedman's technique: * $p < 0.01$; ** not significant.

[b] Insulin release was measured in 30-μl aliquots.

ucts on B-cells were kept as low as possible. With a mean DNA content of 20 ng per mouse islet (Panten, unpublished observations), the mean insulin release caused by α-ketoisocaproic acid at a maximally effective concentration was 9.0 ng/μg of DNA per minute (Fig. 2). The response to 5 mM α-ketoisocaproic acid amounted to about 37% of the latter rate. A similar concentration-response relationship was seen in the microincubation system in which 5 mM α-ketoisocaproic acid induced 35% of the maximal effect (Fig. 3). The maximal secretory capacity of α-ketoisocaproic acid (3.2 ng/μg of DNA per min), however, was only 36% of that seen in the perifusion system. This may result from inhibition of insulin release by accumulation of secretory products. Therefore, we compared insulin secretion and metabolic parameters of islets incubated under identical conditions.

Elimination of metabolites of α-ketoisocaproic acid as trigger intermediates is facilitated by penetration of the extramitochondrial metabolites into cells. The extramitochondrial metabolites L-leucine and isovalerate have been demonstrated to enter islet cells (12, 18). From studies on transport and/or oxidation of α-hydroxymonocarboxylic acids (19), aldehydes (20, 21), and alcohols (22) in other tissues it follows that L-α-hydroxyisocaproic acid, isovaleraldehyde, and isopentanol penetrate plasma membranes. Reduction of α-ketoisocaproic acid by lactate dehydrogenase has been described (23, 24). In case this reaction takes place in islets, it does not mediate the insulin-releasing property of α-ketoisocaproic acid, because L-α-hydroxyisocaproic acid was only marginally effective (Table 1). Previous arguments against L-leucine as mediator of the α-ketoisocaproic acid-induced insulin release (5) are supported by the small effects of a high leucine concentration in the present experiments (Table 1). Preliminary data in the literature reported on an extramitochondrial enzyme which degraded α-ketoisocaproic acid more rapidly than α-ketoisovaleric acid and without necessity of the cofactors NAD and CoA (25–27), but the putative decarboxylation in the extramitochondrial space (Fig. 1) does not produce insulin secretagogues (see Results).

Therefore, only intramitochondrial degradation of α-ketoisocaproic acid could provide insulin-releasing signals (Fig. 1). Branched-chain keto acid dehydrogenase, following enzymes, and the citric acid cycle yield NAD(P)H, reduced flavoproteins, and CoA esters typical of α-ketoisocaproic acid. Twenty millimolar α-ketoisovaleric acid more than doubled insulin release induced by 5 mM α-ketoisocaproic acid (Table 4), although decarboxylation of α-ketoisocaproic acid was inhibited by 47% with ob/ob mouse islets (Table 5). Oxidation of [2-^{14}C]α-ketoisocaproic acid (5 mM), which measures the degradation of isovaleryl-CoA and following CoA compounds, was inhibited 34% by α-ketoisovalerate (Table 5). Therefore, α-ketoisovalerate probably lowered the B-cell content of CoA esters typical of α-ketoisocaproic acid metabolism [islets from ob/ob mice contain more than 90% B-cells (28)]. It is concluded that those CoA esters as such do not initiate insulin secretion. Enhancement of intramitochondrial production of reducing equivalents remains as the only mechanism by which metabolism of α-ketoisocaproic acid could supply releasing signals.

This aspect of the recognition mechanism may be extended to other fuels, provided that later steps of α-ketoisocaproic acid- and glucose-induced insulin release are similar. In a previous study, α-ketoisocaproic acid (5–25 mM) caused a 6- to 25-fold increase of cyclic AMP accumulation by rat islets, suggesting a different mode of action of α-ketoisocaproic acid and glucose in stimulating insulin secretion (11). The present investigation did not confirm these dramatic effects: α-ketoisocaproic acid (15–20 mM) only doubled islet cyclic AMP accumulation. Because of the limitation in temporal resolution, the changes in cyclic AMP accumulation in the present investigation can be related only to the second phase of insulin release. The small cyclic AMP response which requires extracellular Ca^{2+} (Table 6) resembles glucose-induced elevation of cyclic AMP in the islets (29). Thus, there is no convincing evidence for the view that α-ketoisocaproic acid and glucose stimulate insulin secretion by different mechanisms.

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