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Mechanism of the Insulin-Releasing Action of α-Ketoisocaproate and Related α-Keto Acid Anions

Henrike Heissig, Karin A. Urban, Katja Hastedt, Bernd J. Zünkler, and Uwe Panten

Institute of Pharmacology and Toxicology, Technical University of Braunschweig, Braunschweig, Germany (H.H., K.A.U., K.H., U.P.); and Federal Institute for Drugs and Medical Devices, Bonn, Germany (B.J.Z.)

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ABSTRACT

α-Ketoisocaproate directly inhibits the ATP-sensitive K^+ channel (K_{ATP} channel) in pancreatic β-cells, but it is unknown whether direct K_{ATP} channel inhibition contributes to insulin release by α-ketoisocaproate and related α-keto acid anions, which are generally believed to act via β-cell metabolism. In membranes from HIT-T15 β-cells and COS-1 cells expressing sulfonylurea receptor 1, α-keto acid anions bound to the sulfonylurea receptor site of the K_{ATP} channel with affinities increasing in the order α-ketoisovalerate < α-ketovalerate < α-ketoisocaproate < α-ketocaproate < β-phenylpyruvate. Patch-clamp experiments revealed a similar order for the K_{ATP} channel-inhibitory potencies of the compounds (applied at the cytoplasmic side of inside-out patches from mouse β-cells). These findings were compared with the insulin secretion stimulated in isolated mouse islets by α-keto acid anions (10 mM). When all K_{ATP} channels were closed by the sulfonylurea glipizide, α-keto acid anions amplified the insulin release in the order β-phenylpyruvate < α-ketoisovalerate < α-ketovalerate ≈ α-ketocaproate < α-ketoisocaproate. The differences in amplification apparently reflected special features of the metabolism of the individual α-keto acid anions. In islets with active K_{ATP} channels, the first peak of insulin secretion triggered by α-keto acid anions was similar for α-ketoisocaproate, α-ketocaproate, and β-phenylpyruvate but lower for α-ketovalerate and insignificant for α-ketoisovalerate. This difference from the above orders indicates that direct K_{ATP} channel inhibition is not involved in the secretory responses to α-ketoisovalerate and α-ketovalerate, moderately contributes to initiation of insulin secretion by α-ketoisocaproate and α-ketocaproate, and is a major component of the insulin-releasing property of β-phenylpyruvate.

The metabolism of glucose and some other fuels in the pancreatic β-cell provides signals for rapid stimulation of insulin secretion (Henquin, 2000; MacDonald et al., 2005). It is believed that major signals are an increase in cytosolic ATP and a decrease in cytosolic ADP caused by activation of the mitochondrial energy metabolism (Henquin, 2000). These changes in cytosolic nucleotides inhibit the ATP-sensitive K^+ channel (K_{ATP} channel) in the β-cell plasma membrane (Aguilar-Bryan and Bryan, 1999). The channel inhibition depolarizes the membrane, voltage-dependent calcium channels are opened, and the resulting increase in the cytosolic free Ca^{2+} concentration triggers the exocytosis of insulin. As soon as insulin release is initiated, the secretory response is enhanced by an amplifying pathway requiring the metabolism of the fuel secretagogue (Henquin, 2000). The ATP/ADP ratio in the β-cell cytosol has been suggested to serve as amplification signal.

α-Ketoisocaproate (4-methyl-2-oxopentanoate), the transamination product of L-leucine, and some related α-keto acid anions (α-ketocaproate, α-ketovalerate, and β-phenylpyruvate) stimulate insulin secretion by pancreatic islets in the absence of any other fuel or secretagogue (Panten et al., 1972; Matschinsky et al., 1973; Hutton et al., 1980; Lenzen and Panten, 1980). This effect requires millimolar extracellular concentrations (>10-fold higher than the plasma concentrations of α-ketoisocaproate in healthy humans) (Schauder et al., 1985), which lead to millimolar concentrations in the cytosol and mitochondria of β-cells (Hutton et al., 1979; Malaisse et al., 1983; Hutson et al., 1990). At these concentrations, transamination of α-keto acid anions with endogenous glutamate and glutamine enhances the availability of α-ketoglutarate in the β-cell mitochondria and thereby increases the capacity of the citrate cycle (Hutton et al., 1979; Malaisse et al., 1981, 1983; Lenzen et al., 1984, 1986; MacDonald et al., 2005). The resulting promotion of the oxidation of exogenous and endogenous fuels rapidly activates the β-cell energy metabolism (Panten et al., 1972; Hutton et al., 1979;

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ABBREVIATIONS: K_{ATP} channel, ATP-sensitive K^+ channel; SUR, sulfonylurea receptor; K_{IR}, potassium inward rectifier channel; EGFP, enhanced green fluorescent protein; P1075, *N*-cyano-*N*′-(1,1-dimethylpropyl)-*N*′′-3-pyridylguanidine.

Lenzen and Panten, 1980; Duchen et al., 1993). These findings and the α-ketoisocaproate-induced inhibition of K_{ATP} channels in intact β-cells (Ashcroft et al., 1987) led to the view that α-ketoisocaproate and related α-keto acid anions trigger insulin release by enhancing the ATP production in β-cell mitochondria.

This long-standing view of the insulin-releasing action of α-ketoisocaproate and related α-keto acid anions has been questioned by the observation of a direct inhibitory effect of α-ketoisocaproate on the β-cell K_{ATP} channel (Bränström et al., 1998). The authors concluded that insulin release in response to α-ketoisocaproate might result not only from enhanced mitochondrial ATP production but also from direct inhibition of the K_{ATP} channel. In pancreatic islets, the transamination inhibitor aminooxyacetate strongly inhibited α-ketoisocaproate–induced increase in cytosolic Ca^{2+} and insulin secretion (Malaisse et al., 1982; Gao et al., 2003). However, these findings do not rule out a significant contribution of direct K_{ATP} channel inhibition because it is unknown whether indirect (metabolic) K_{ATP} channel inhibition by α-ketoisocaproate is sufficient to initiate a strong insulin release.

The present study aimed at investigating the role of direct K_{ATP} channel inhibition in the stimulation of insulin secretion by α-ketoisocaproate and related α-keto acid anions. Therefore, we examined the mechanism of this direct inhibition and compared the results with the capacities of the α-keto acid anions to release insulin from β-cells with or without active K_{ATP} channels.

Materials and Methods

Chemicals. Sigma/Fluka (Taufkirchen, Germany) provided α-ketoisocaproate (4-methyl-2-oxopentanoate), α-ketocaproate (2-oxohexanoate), α-ketoisovalerate (3-methyl-2-oxobutyrate), α-ketovalerate (2-oxopentanoate), β-phenylpyruvate (2-oxo-3-phenylpropionate), pyruvate, and *n*-hexanoate as sodium salts and 3-methylbutyric acid, *n*-pentanoic acid, 4-methylpentanoic acid, 3-phenylpropionic acid, and aminooxyacetic acid hemihydrochloride. α-Ketoisocaproic acid, glipizide, and meglitinide, were from Roth (Karlsruhe, Germany), Pfizer (Karlsruhe, Germany), and Aventis (Strasbourg, France), respectively. All other chemicals and radioactively labeled compounds were obtained from sources described elsewhere (Panten et al., 1989; Meyer et al., 1999).

Electrophysiological Experiments. COS-7 cells were plated at a density of 2×10^5 cells per 35-mm dish and cultured as described previously (Zünkler et al., 2000). The cells were transiently transfected with the pcDNA3 vector containing the coding sequence of $K_{IR}6.2\Delta C26$ (provided by Dr. F. Ashcroft, Oxford University, Oxford, England, UK) and of enhanced green fluorescent protein (EGFP). The plasmid concentrations were 5 and 0.5 μg per 35-mm dish (containing 1 ml of culture medium) for $K_{IR}6.2\Delta C26$ and EGFP, respectively. Transfections were performed as described previously (Meyer et al., 1999). Single-channel currents were studied 48 to 72 h after transfection. Inside-out membrane patches were used only from cells expressing EGFP (visualization aided by a laser-scanning confocal imaging system) (Zünkler et al., 2000).

Albino mice of both genders (NMRI, 9–13 weeks old, fed ad libitum) were used. As described previously (Panten et al., 1989), pancreatic islets were isolated by collagenase digestion in basal medium (containing 2 mg/ml albumin) supplemented with 5 mM glucose. The islets were dissociated into single cells by shaking in a solution without Ca^{2+} (Lernmark, 1974). The cells were cultured (in the presence of 10 mM glucose) on 35-mm dishes as detailed previously (Schwanstecher et al., 1994). Single-channel currents were studied after 24 to 72 h of culture.

A standard patch-clamp technique was used in the inside-out configuration as described previously with minor modifications (Meyer et al., 1999). Pipettes were pulled from borosilicate glass (Hilgenberg, Malsfeld, Germany), and pipette resistances ranged between 3 and 7 MΩ (experiments with COS-7 cells) or between 5 and 7 MΩ (experiments with β-cells) when filled with pipette solution which contained 146 mM KCl, 2.6 mM $CaCl_2$, 1.2 mM $MgCl_2$, and 10 mM HEPES titrated to pH 7.40 with KOH. The pipette potential was held constant at +60 mV (membrane potential, −60 mV; experiments with COS-7 cells) or at +50 mV (membrane potential, −50 mV; experiments with β-cells), and inward membrane currents flowing from the pipette to the bath solution were recorded (indicated by downward deflections). The bath solution contained 140 mM KCl, 1 mM $MgCl_2$, 10 mM EGTA, 2 mM $CaCl_2$, and 5 mM HEPES titrated to pH 7.15 with KOH (experiments with COS-7 cells) or to pH 7.30 with KOH (experiments with β-cells). Bath solution supplemented with 15 mM α-ketoisocaproate (experiments with COS-7 cells) was prepared by substituting 15 mM α-ketoisocaproic acid for equimolar amounts of KCl and titrating pH to 7.15 with KOH. Sodium salts of test compounds were directly dissolved in the bath solution. When the bath solution was supplemented with ATP or ADP, the free Mg^{2+} concentration was held close to 0.7 mM by adding the appropriate amounts of $MgCl_2$ (calculated as described by Schwanstecher et al., 1994). The bath was perfused at 2 ml/min, and approximately 30 s was needed for the exchange of the bath solution. All experiments were performed at room temperature (20–22°C).

Current signals were filtered at 2 kHz with a Bessel filter, digitized with an A/D converter, and stored on video tape. Stored records were displayed with a digital plotter or a chart recorder. Stored data were digitized at 10 kHz using an adapter (Digidata 1200 Interface; Axon Instruments Inc., Union City, CA) and analyzed with the pCLAMP 6.0 software (Axon Instruments).

For experiments with COS-7 cells, channel activity ($N \times P_o$) was calculated as $N \times P_o = 1/T \times \Sigma\, n_i \times t_i$, where N was the number of available channels in the patch (estimated as the maximum number of open channels), P_o was the open probability of a single channel, t_i was the time spent at each current level n_i, and the total recording time (T) was usually 20 to 30 s. The channel activity during the test period was compared with the mean of the channel activity during the control periods before and after the test period.

In experiments with islet cells, patches from non-β-cells probably contributed only a minor proportion to the results of our study. First, islet cell suspensions prepared from mouse islets by the method also applied in our study contained only <2 or <0.5% of δ- or PP-cells, respectively (Barg et al., 2000). Second, with reported sizes and K_{ATP} channel densities of α- and β-cells (Barg et al., 2000), and with calculated sizes of membrane patches in our pipettes (5–7 MΩ) (Sakmann and Neher, 1983), inside-out patches from α- or β-cells are expected to contain approximately 1 or 20 to 30 channels, respectively, but the number of active K_{ATP} channels observed in our patches ranged between 3 and 65 (mean value = 22 ± 1, n = 162). All α-keto acid anions were tested in the presence of 1 mM ADP, because this enabled the complete closure of K_{ATP} channels by sulfonylureas and analogs, thereby facilitating the analysis of concentration-response relationships and avoiding channel closure by direct effects on $K_{IR}6.2$ (Schwanstecher et al., 1994; Gribble et al., 1997). Before and after each test period, there were control periods with bath solution containing 1 mM ADP. To consider channel run-down, the channel current during the test period was normalized to the mean of the channel current during the two control periods. Each control period was preceded or followed, respectively, by a period with bath solution containing 1 mM ATP. The latter periods indicated the baseline and the activity of a channel with a lower single-channel current amplitude (approximately 1.2 pA) than that of the K_{ATP} channel (3.6 pA). The 1.2-pA channel was seen in many patches and did not seem to be altered by ATP or the applied test compounds. All

α-keto acids were applied as sodium salts because some compounds were only available as sodium salts. To consider the influences of Na^+ on the channel currents, the Na^+ concentration was held constant during the experiment by the addition of NaCl to the bath solutions containing only ATP or ADP. Only one concentration of α-keto acid anion or *n*-hexanoate was tested per patch. The tested compounds did not change the single-channel current amplitudes of the K_{ATP} channel. Because of the large number of K_{ATP} channels in the inside-out membrane patches from mouse β-cells, the mean current flowing through all open K_{ATP} channels (I) was measured. I correlates with the channel activity ($N \times P_o$) according to the equation $I = N \times P_o \times i$, where i is the mean single K_{ATP} channel current amplitude (3.6 pA). Normalized channel current was calculated as Channel current (%) = $(I_{test} - I_{ATP}) \times 100/(I_{control} - I_{ATP})$, where I_{test} was the mean current during the test period, $I_{control}$ was the mean current during the two control periods, and I_{ATP} was the mean current during the two periods with ATP. I_{ATP} was included in the calculation to take into account the activity of the 1.2-pA channel. The mean value for I_{ATP} amounted to 6.8 ± 0.5% of $I_{control}$ (n = 162). Data sampling was usually performed during the last 60 to 90 s before medium change.

Measurement of Insulin Secretion. Batches of 50 islets (for animals and isolation, see above) were perifused at 0.9 ml/min and 37°C with basal medium containing 2 mg/ml albumin (other additions are detailed under *Results*) as described previously (Panten et al., 1989). Supplementation of medium with glipizide or meglitinide was performed by the addition of appropriate amounts of stock solutions in 50 mM NaOH. Sodium salts were directly dissolved in the media. The experiments began with a control period of 60-min duration which was followed by a test period lasting 44 min. The insulin content of 1- to 4-min fractions was determined by enzyme-linked immunosorbent assay (Mercodia, Uppsala, Sweden) with rat insulin as reference. The tested compounds did not influence the assay. In the figures, the values of the secretory rates are depicted in the middle of the sampling intervals. The rate of insulin secretion is expressed as a percentage of the secretion rate at the end of the control period.

Binding Experiments. Culture of HIT-T15 cells (SV-40 transformed hamster β-cells) and COS-1 cells, transient transfections of COS-1 cells (clones provided by Dr. J. Bryan), membrane preparations, and measurement of ligand binding to the membranes were performed as described previously (Meyer et al., 1999). For measurement of [^{3}H]glibenclamide binding to SUR1, resuspended membranes were incubated for 1 h at room temperature (20–22°C) in 1 ml of Tris-HCl buffer, pH 7.4 (185 mM Tris), containing [^{3}H]glibenclamide (0.3 nM; final concentration) and test substances (final concentrations are indicated under *Results*). Nonspecific binding was defined by 100 nM glibenclamide. For measurement of [^{3}H]P1075 binding to SUR2B, resuspended membranes were incubated for 1 h at room temperature (20–22°C) in 0.5 ml of Tris-HCl buffer, pH 7.4 (140 mM Tris), containing (final concentrations) [^{3}H]P1075 (3 nM), $MgCl_2$ (1 mM), ATP (0.1 mM), and test substances (final concentrations indicated under *Results*). Nonspecific binding was defined by 100 μM pinacidil. In both assay types, sodium salts were directly dissolved in the Tris buffers, whereas acids were first mixed with appropriate amounts of NaOH solution and then added to the Tris buffers (usually pH adjustment was not necessary).

Treatment of Results. Values are presented as means ± S.E. Analysis of relations between concentration of test compound and K_{ATP} channel current or specific binding and calculation of K_d values were performed as described previously (Meyer et al., 1999). The Kruskal-Wallis test was applied, followed by analysis of differences between interesting groups using the Mann-Whitney U test (two-tailed), together with the Bonferroni-Holm procedure for multiple comparisons. At $p < 0.05$, significance was assumed.

Results

Effects of α-Ketoisocaproate on K_{IR}6.2ΔC26 Channel Activity. The K_{ATP} channels of β-cells are composed of two proteins, a subunit (K_{IR}6.2) forming the K^+-selective pore, and a regulatory subunit (SUR1) (Aguilar-Bryan and Bryan, 1999). ATP closes the K_{ATP} channel by binding to K_{IR}6.2, whereas MgADP induces channel opening by binding to SUR1. Insulin-releasing sulfonylureas (e.g., glibenclamide and glipizide) and their analogs (e.g., meglitinide) close the K_{ATP} channel by binding to SUR1 (Aguilar-Bryan and Bryan, 1999; Meyer et al., 1999). K_{IR}6.2 forms only very few functional K^+ channels when expressed in the absence of SUR1. But truncation of the carboxyl terminus of K_{IR}6.2 by the terminal 26 amino acids (K_{IR}6.2ΔC26) produces high K_{ATP} channel activity in the absence of SUR1 (Tucker et al., 1997). In inside-out patches of COS-7 cells cotransfected with K_{IR}6.2ΔC26 and EGFP cDNA, ion channels with an amplitude of 5.4 ± 0.1 pA (n = 5) at a membrane potential of −60 mV were observed. In most inside-out patches, run-down of channel activity occurred within a few minutes after excision of the patch (Fig. 1). The experiment in Fig. 1 and four similar experiments did not provide evidence for inhibition of the channel activity by α-ketoisocaproate. Channel activity in the presence of 15 mM α-ketoisocaproate was 96.8 ± 8.6% of the channel activity during the control periods. In contrast, 0.3 mM ATP inhibited the channel activity in each single experiment. Channel activity in the presence of 0.3 mM ATP

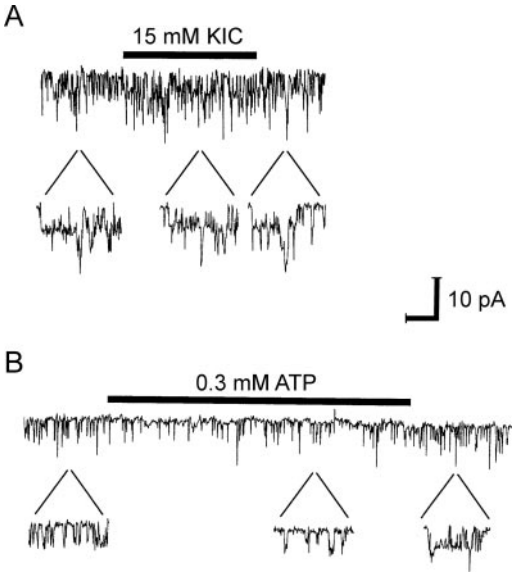

Fig. 1. Effects of α-ketoisocaproate and ATP on K_{IR}6.2ΔC26 channel activity in an inside-out patch of a transfected COS-7 cell. The upper current traces in A and B show recordings of K_{IR}6.2ΔC26 channel activity from the same membrane patch. Note the lower channel activity in B compared with A caused by channel rundown. Segments of channel activities from the continuous traces are shown below each trace on an expanded time scale. The horizontal scale bar on the middle right corresponds to 50 s for the continuous traces and to 100 ms for the expanded segments. The horizontal bars above the continuous traces indicate the application of intracellular solution containing 15 mM α-ketoisocaproate (KIC) or 0.3 mM ATP by the bath. A, 15 mM α-ketoisocaproate (KIC) had no clear effect on K_{IR}6.2ΔC26 channel activity. Values for $N \times P_o$ were the following: 0.190 (control before α-ketoisocaproate application); 0.281 (15 mM α-ketoisocaproate); and 0.331 (control after α-ketoisocaproate application). B, 0.3 mM ATP decreased K_{IR}6.2ΔC26 channel activity by 57%. Values for $N \times P_o$ were the following: 0.063 (control before ATP application); 0.049 (0.3 mM ATP); and 0.164 (control after ATP application).

was 41.4 ± 5.9% of the channel activity during the control periods.

Binding of α-Ketoisocaproate and Related Carboxylic Acid Anions to Sulfonylurea Receptors. The failure of α-ketoisocaproate to reduce $K_{IR}6.2\Delta C26$ channel activity suggested that inhibition of K_{ATP} channel activity resulted from interaction of α-ketoisocaproate with SUR1. We therefore measured the binding of α-ketoisocaproate and related carboxylic acid anions to native SUR1 (in membranes from HIT-T15 β-cells expressing both SUR1 and $K_{IR}6.2$) and to transiently expressed SUR1 or SUR2B (in membranes from COS-1 cells expressing no $K_{IR}6.2$). SUR2B represents the regulatory subunit of the K_{ATP} channel in smooth muscle (Aguilar-Bryan and Bryan, 1999) and was included in the experiments to give information on the selectivity of receptor binding of α-ketoisocaproate and related carboxylic acid anions.

Competitive inhibition assays showed that 60 to 100 mM concentrations of β-phenylpyruvate inhibited specific ^{3}H-ligand binding by approximately 90% (Fig. 2). Therefore, relations between the concentrations of carboxylic acid anions and specific binding were analyzed assuming complete inhibition by maximally effective concentrations. The IC_{50} values increased in the order β-phenylpyruvate < α-ketoisocaproate < α-ketoisovalerate (Fig. 2). NaCl concentrations up to 200 mM did not inhibit ^{3}H-ligand binding to SUR1 and inhibited ^{3}H-ligand binding to SUR2B by approximately 10%. By use of the experimental design shown in Fig. 2, IC_{50} values were determined for a series of carboxylic acid anions related to α-ketoisocaproate. The K_d values (Table 1) calculated from the IC_{50} values did not reveal major differences between the binding affinities ($1/K_d$) of α-keto acid anions and the binding affinities of their corresponding carboxylic acid anions without keto group. There were also no major differences between the binding affinities for SUR1 in the presence or absence of $K_{IR}6.2$. The binding affinities for SUR2B were nearly always slightly higher than those for SUR1. However, these differences might have been caused by the fact that no correction has been made for nonspecific effects on SUR2B binding, as revealed by high NaCl concentrations (see above).

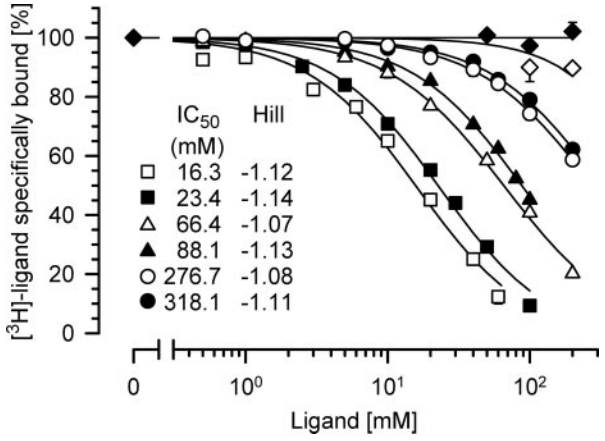

Fig. 2. Sulfonylurea receptor binding of β-phenylpyruvate, α-ketoisocaproate, and α-ketoisovalerate. In membranes from COS-1 cells transiently expressing hamster SUR1 or rat SUR2B, the effects of β-phenylpyruvate (□, ■), α-ketoisocaproate (△, ▲), α-ketoisovalerate (○, ●), and Cl^- (◇, ◆) on specific ^{3}H-ligand binding were measured. For binding to recombinant SUR1 (closed symbols), the ^{3}H-ligand was [^{3}H]glibenclamide (0.3 nM); for binding to recombinant SUR2B (open symbols), the ^{3}H-ligand was [^{3}H]P1075 (3 nM). Results are expressed as a percentage of control (absence of displacing drug). Symbols are means (with S.E. shown when larger than symbols) from four to five separate binding experiments. Analysis revealed the indicated IC_{50} values and Hill coefficients.

Effects of α-Ketoisocaproate and Related Carboxylic Acid Anions on K_{ATP} Channel Currents in β-Cells. In inside-out patches of mouse β-cells, K_{ATP} channels (amplitudes of 3.6 pA) and a channel with a lower amplitude (approximately 1.2 pA) were observed (Fig. 3). The current traces revealed pronounced rundown of K_{ATP} channel activity. Similar rundown occurred in nearly all experiments and was considered by including control periods before and after the test period (for calculation, see *Materials and Methods*). The example in Fig. 3A indicates that in the presence of 1 mM ADP, 20 mM β-phenylpyruvate reduced the K_{ATP} channel current by 97.5% to a level as low as in the sole presence of 1 mM ATP. Application of 20 mM *n*-hexanoate (Fig. 3B) reduced the K_{ATP} channel current by 63.5%. In addition, 20 mM *n*-hexanoate induced a reversible shift of the baseline, indicating the development of inward currents (flowing from the pipette to the bath solution). Similar shifts of baseline took place in many experiments with *n*-hexanoate but never in experiments with α-keto acid anions. We believe the shifts in baseline to have been caused by the effects of undissociated *n*-hexanoic acid on the lipid phase of the patch mem-

TABLE 1

K_d values of α-ketoisocaproate and related carboxylic acid anions for sulfonylurea receptor binding

The listed K_d values (means ± S.E.) and Hill coefficients (means) were obtained from four to six independent competition curves. K_d values were calculated according to Cheng and Prusoff (1973) from the IC_{50} values of individual binding experiments with membranes from HIT-T15 cells expressing SUR1 (HIT-SUR1) or COS-1 cells transiently expressing hamster SUR1 (SUR1) or rat SUR2B (SUR2B).

Ligand	HIT-SUR1		SUR1		SUR2B	
	K_d	Hill	K_d	Hill	K_d	Hill
	mM		*mM*		*mM*	
α-Ketoisovalerate	N.M.	N.M.	203 ± 11	−1.12	221 ± 21	−1.11
3-Methylbutyrate	253 ± 23	−1.11	279 ± 13	−1.16	207 ± 13	−1.10
α-Ketovalerate	98.8 ± 6.4	−1.02	85.9 ± 9.0	−1.06	55.6 ± 1.8	−1.05
n-Pentanoate	148 ± 6.5	−1.07	95.3 ± 1.7	−1.05	81.0 ± 1.8	−1.07
α-Ketoisocaproate	46.9 ± 1.1	−1.27	57.2 ± 4.0	−1.11	52.9 ± 2.1	−1.07
4-Methylpentanoate	45.6 ± 1.0	−1.13	53.1 ± 0.6	−1.05	25.2 ± 1.0	−1.05
α-Ketocaproate	24.9 ± 0.9	−1.08	25.6 ± 0.7	−1.05	21.7 ± 1.8	−1.09
n-Hexanoate	42.1 ± 1.1	−1.04	29.7 ± 0.6	−1.06	25.2 ± 0.8	−1.19
β-Phenylpyruvate	12.8 ± 0.4	−1.06	15.2 ± 0.3	−1.10	12.7 ± 0.9	−1.13
3-Phenylpropionate	18.5 ± 0.7	−0.96	19.2 ± 0.9	−1.00	13.1 ± 1.0	−1.11

N.M., not measured.

brane. At pH 7.40 and a total concentration of 20 mM, the concentration of undissociated *n*-hexanoic acid is 50 μM (pK_a for *n*-hexanoic acid = 4.8), whereas the concentrations of undissociated α-keto acids are negligible (pK_a for pyruvate = 2.5).

β-Phenylpyruvate (20 mM) nearly completely inhibited K_{ATP} channel current (Fig. 4). Therefore, relations between the concentrations of carboxylic acid anions and channel current were analyzed, assuming complete inhibition by maximally effective concentrations. The potencies ($1/IC_{50}$) for channel inhibition increased in the order α-ketoisovalerate < α-ketovalerate < α-ketoisocaproate < *n*-hexanoate < α-ketocaproate < β-phenylpyruvate. Application of NaCl (up to 40 mM) during the test periods instead of sodium salts of carboxylic acids did not reduce the K_{ATP} channel current (Fig. 4).

Insulin-Releasing Effects of α-Ketoisocaproate, Related Carboxylic Acid Anions, and Meglitinide. After perifusion of islets for 60 min in the absence of any fuel or secretagogue, the rate of insulin secretion was 4.6 ± 0.2 pg of insulin/min/islet (n = 56, all experiments in Fig. 5). In groups of 6 to 10 experiments, the secretion rates at the end of the control period varied considerably because of differences in islet size. Therefore, secretory rates were normalized to the rates at the end of the control period. All tested α-keto acid anions (10 mM) induced an initial peak of insulin release at minute 62.5 (Fig. 5, A and B). Whereas the secretory rates at minute 62.5 were similar for α-ketoisocaproate, α-ketocaproate, and β-phenylpyruvate, the rate for α-ketovalerate was significantly lower ($p < 0.02$ in comparison with α-ketocaproate; $p < 0.05$ in comparison with α-ketoisocaproate or β-phenylpyruvate). α-Ketoisovalerate caused only a very small peak of insulin secretion ($p < 0.02$ in comparison with the rate at minute 62.5 in the absence of test compound) (Fig. 5B). The tested α-keto acid anions (10 mM) differed also in the secretory profile after the initial peak of insulin release (Fig. 5, A and B). Some compounds produced a second phase of insulin secretion which was strong and sustained (α-ketoisocaproate), strong, but gradually decreasing from minutes 78 to 102 (α-ketocaproate), or weak (α-ketovalerate). β-Phenylpyruvate did not induce a second phase of insulin release. From minutes 82 to 102, the average secretory rate for β-phenylpyruvate was lower ($p < 0.01$) than in the case of α-ketovalerate (Fig. 5A). Compared with the corresponding secretory rates in the absence of test compound, the sulfonylurea analog meglitinide (0.1 mM) slightly enhanced the secretory rate at minute 62.5 ($p < 0.02$) and induced a prolonged insulin release (average secretory rate from minutes 82 to 102, $p < 0.02$) (Fig. 5B). *n*-Hexanoate (10 mM) did not stimulate insulin secretion (results not shown).

Insulin-Releasing Effects of α-Ketoisocaproate and Related Carboxylic Acid Anions Not Induced by K_{ATP} Channel Inhibition. In isolated mouse islets, all K_{ATP} channels of which were closed by maximally effective concentrations of sulfonylureas, α-ketoisocaproate strongly amplified the insulin secretion (Panten et al., 1988). To compare the amplifying effects of α-keto acid anions, we performed perfusion experiments with islets exposed to the sulfonylurea glipizide at a concentration (2.7 μM) completely inhibiting the K_{ATP} channels of β-cells (Panten et al., 1989). Complete

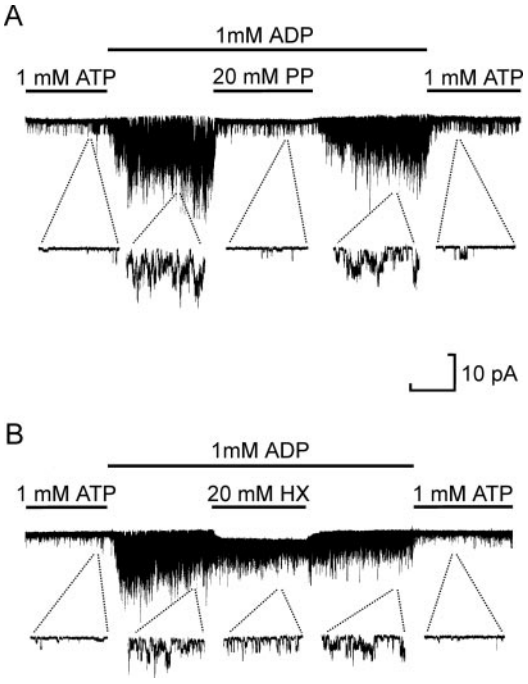

Fig. 3. Effects of β-phenylpyruvate (A) and *n*-hexanoate (B) on K_{ATP} channel currents in inside-out patches of mouse pancreatic β-cells. Free Mg^{2+} (0.7 mM) was always present in the solutions applied at the cytoplasmic membrane side. Segments of channel activities from the continuous traces are shown below each trace on an expanded time scale. The horizontal scale bar on the middle right corresponds to 1 min for the continuous traces and to 1 s for the expanded segments. The horizontal bars above the current traces obtained from inside-out patches indicate the application of intracellular solution containing 1 mM ATP, 1 mM ADP, 1 mM ADP plus 20 mM sodium β-phenylpyruvate (PP), or 1 mM ADP plus 20 mM sodium *n*-hexanoate (HX) by the bath. The sodium concentrations in the solutions containing only ATP or ADP were made equal to the sodium concentrations in the solutions containing β-phenylpyruvate or *n*-hexanoate by adding NaCl.

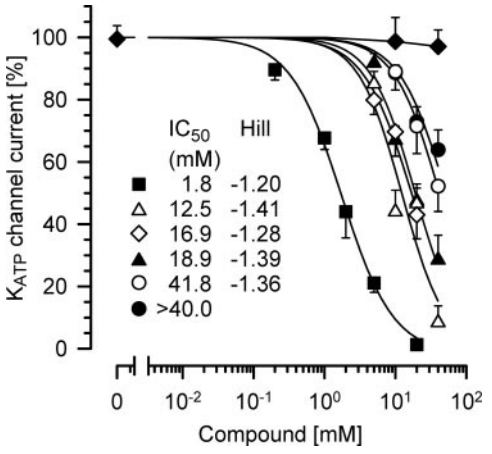

Fig. 4. Relationships between K_{ATP} channel currents and concentrations of carboxylic acid anions. The test compounds were β-phenylpyruvate (■), α-ketocaproate (△), α-ketoisocaproate (▲), *n*-hexanoate (◇), α-ketovalerate (○), α-ketoisovalerate (●), and Cl^- (◆). By use of the experimental design shown in Fig. 3, K_{ATP} channel current during the test period was normalized to K_{ATP} channel current during the control periods (presence of 1 mM ADP, absence of test compound) before and after the application of each concentration of test compound. K_{ATP} channel current during the test and control periods was corrected for channel current not suppressed by 1 mM ATP (see *Materials and Methods*). Symbols are means (with S.E. shown when larger than symbols) from 4 to 10 experiments. Analysis revealed the indicated IC_{50} values and Hill coefficients. Data for α-ketocaproate and α-ketoisocaproate are taken from the dissertation of Schmeling (2004) and have been obtained by the same method as applied in the present study.

K_{ATP} channel block in islets perifused with 2.7 μM glipizide was verified by the finding that after perifusion for 60 min with 2.7 μM glipizide, transition to 20 μM glipizide did not enhance insulin secretion (results not shown).

After perifusion of mouse islets for 60 min in the presence of 2.7 μM glipizide but in the absence of any substrate, the insulin secretion rate was 14.4 $\pm$ 1.2 pg of insulin/min/islet (n = 52, all experiments in Fig. 6A). This rate was significantly higher ($p < 0.001$) than the corresponding rate in the absence of any fuel or secretagogue (4.6 $\pm$ 0.2 pg of insulin/min/islet, see above). All tested α-keto acid anions (10 mM) significantly enhanced insulin secretion within 3 to 4 min (Fig. 6A). Secretory maxima were reached after different periods (14 min for α-ketoisocaproate and α-ketocaproate, 3 to 4 min for α-ketovalerate, α-ketoisovalerate, and β-phenylpyruvate). After the maxima, the secretion rates decreased slowly in the case of α-ketoisocaproate, α-ketocaproate, and α-ketovalerate or rapidly in the case of α-ketoisovalerate and β-phenylpyruvate. At the end of the test period, insulin secretion was still higher in the presence than in the absence of α-ketoisovalerate ($p < 0.05$ for comparison with the corresponding secretory rate in the absence of test compound). However, β-phenylpyruvate did not enhance insulin secretion during the last 20 min of the test period (Fig. 6A). The insulin released during the total test period increased in the order β-phenylpyruvate < α-ketoisovalerate < α-ketovalerate $\approx$ α-ketocaproate < α-ketoisocaproate, and the following significances were calculated for comparisons of total insulin release: β-phenylpyruvate versus α-ketoisovalerate, $p < 0.05$; α-ketoisovalerate versus α-ketovalerate, $p < 0.02$; α-ketovalerate versus α-ketocaproate, $p > 0.05$; α-ketocaproate versus α-ketoisocaproate, $p < 0.01$. Pyruvate (20 mM) and n-hexanoate (10 mM) did not stimulate insulin secretion (n = 5–6, experimental design as in Fig. 6A, results not shown). Pyruvate enters the β-cells as indicated by the high rate of pyruvate decarboxylation in mouse pancreatic islets (Lenzen and Panten, 1980).

Because the transamination inhibitor aminooxyacetate strongly inhibited insulin secretion induced by α-ketoisocaproate in islets with active K_{ATP} channels (Malaisse et al., 1982), we wanted to know whether aminooxyacetate exerted

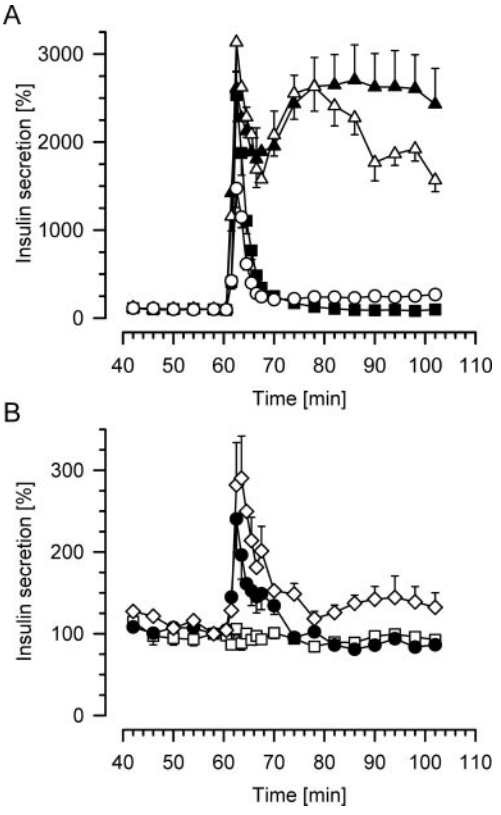

Fig. 5. Effects of α-keto acid anions and meglitinide on the kinetics of insulin secretion by mouse pancreatic islets. Values in the curves are means (with S.E. shown when larger than symbols, some error bars omitted for clarity) of results from 8 to 10 (A) or 6 to 7 (B) separate experiments. From zero time to minute 60, the islets were perifused with basal medium. A, from minutes 61 to 104, the islets were perifused with basal medium containing 10 mM concentrations of β-phenylpyruvate (■), α-ketocaproate (△), α-ketoisocaproate (▲), or α-ketovalerate (○). B, from minutes 61 to 104, the islets were perifused with basal medium containing no test compound (controls, □) or with basal medium containing 10 mM α-ketoisovalerate (●) or 0.1 mM meglitinide (◇).

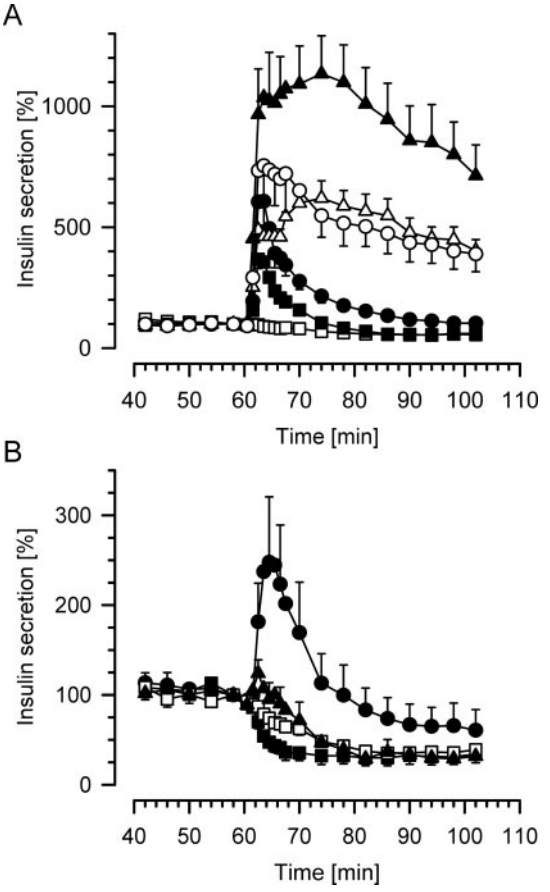

Fig. 6. Effects of α-keto acid anions on the kinetics of insulin secretion by mouse islets exposed to glipizide (A) or glipizide + aminooxyacetate (B). Values in the curves are means (with S.E. shown when larger than symbols, some error bars omitted for clarity) of results from 7 to 10 (A) or 4 to 5 (B) separate experiments. A, from zero time to minute 60, the islets were perifused with basal medium containing 2.7 μM glipizide. From minutes 61 to 104 the islets were perifused with basal medium containing 2.7 μM glipizide (controls, □) or with basal medium containing 2.7 μM glipizide and 10 mM concentrations of β-phenylpyruvate (■), α-ketocaproate (△), α-ketoisocaproate (▲), α-ketoisovalerate (●), or α-ketovalerate (○). B, from zero time to minute 60, the islets were perifused with basal medium containing 2.7 μM glipizide and 5 mM aminooxyacetate. From minutes 61 to 104, the islets were perifused with basal medium containing 2.7 μM glipizide and 5 mM aminooxyacetate (controls, □) or with basal medium containing 2.7 μM glipizide, 5 mM aminooxyacetate, and 10 mM concentrations of β-phenylpyruvate (■), α-ketoisocaproate (▲), or α-ketoisovalerate (●).

a similar effect in islets all K_{ATP} channels of which were closed by glipizide. After perifusion of islets for 60 min with 2.7 μM glipizide plus 5 mM aminooxyacetate the rate of insulin secretion was 14.2 ± 2.1 pg of insulin/min/islet (n = 17, all experiments in Fig. 6B). In the presence of 2.7 μM glipizide plus 5 mM aminooxyacetate, 10 mM α-ketoisocaproate, or 10 mM β-phenylpyruvate did not stimulate insulin release, and 10 mM α-ketoisovalerate was much less effective than in the absence of aminooxyacetate (Fig. 6, A and B).

Discussion

The present study indicates that α-ketoisocaproate and related carboxylic acid anions inhibit K_{ATP} channels by binding to the receptor site for sulfonylureas and their analogs (Figs. 1–4, Table 1). As in the case of sulfonylureas and their analogs (Panten et al., 1989; Schwanstecher et al., 1994; Meyer et al., 1999), Hill coefficients for binding were close to 1, the expression of $K_{IR}6.2$ did not influence the affinities for binding to SUR1, the order of affinities for receptor binding corresponded to the order of potencies for K_{ATP} channel inhibition, and the K_d values for binding were always higher than the corresponding IC_{50} values for K_{ATP} channel inhibition. The latter differences reflect the fact that occupation of one of the four SUR binding sites per channel complex is sufficient for K_{ATP} channel closure (theoretical K_d/IC_{50} ratio = 5.75) (Dörschner et al., 1999). In addition, α-ketoisocaproate and related carboxylic acid anions displayed features characteristic of the sulfonylurea analog meglitinide (Meyer et al., 1999): an α-keto group in the molecules was not essential for the interaction of carboxylic acid anions with the K_{ATP} channels, and the tested carboxylic acid anions did not distinguish between SUR1 and SUR2B (Figs. 2 and 4, Table 1).

The receptor site for sulfonylureas and their analogs is located at the intracellular side of the plasma membrane (Schwanstecher et al., 1994; Ashfield et al., 1999). The extent of K_{ATP} channel inhibition by extracellularly applied carboxylic acid anions is therefore determined by the cytosolic concentrations of the compounds. After transport across the β-cell membrane, the α-keto acid anions tested in the present study are partially converted into the corresponding amino acids by extramitochondrial transaminases (Panten et al., 1972; Hutton et al., 1979; Malaisse et al., 1982, 1983; Lenzen et al., 1984). Transamination and efflux of amino acids out of the β-cells considerably reduce the cytosolic α-keto acid anion concentrations. When rat islets were incubated in the presence of 10 mM concentrations of α-ketoisocaproate or β-phenylpyruvate, concentrations of approximately 2 mM were found in the intracellular water space of the islets (Hutton et al., 1979; Malaisse et al., 1983). The latter concentrations correspond to 3 to 4 mM in the β-cell cytosol, assuming restriction of the α-keto acid anions to the cytosol and the mitochondrial matrix (Dean, 1973). At 3 to 4 mM concentrations, the curves in Fig. 4 indicate that α-ketoisovalerate and α-ketovalerate do not reduce the β-cell K_{ATP} channel current, that α-ketoisocaproate and α-ketocaproate induce a slight (~10%) K_{ATP} channel inhibition, and that β-phenylpyruvate produces a pronounced (~70%) channel inhibition. The failure of 15 mM α-ketoisocaproate to inhibit the $K_{IR}6.2\Delta C26$ channel (see *Results*) argues against direct effects of 3 to 4 mM concentrations of α-keto acid anions on $K_{IR}6.2$. The application of 1 mM ADP at the cytoplasmic side of the inside-out membrane patches (Fig. 4) probably did not affect the inhibitory potency of α-keto acid anions, because ADP did not significantly alter the IC_{50} values of sulfonylureas and analogs for SUR1-mediated inhibition of the β-cell K_{ATP} channel (Schwanstecher et al., 1994; Gribble et al., 1997). The correction of our data for channel rundown (Fig. 4) explains why α-ketoisocaproate displayed a potency for K_{ATP} channel inhibition which was lower (IC_{50} = 18.9 mM, Fig. 4) than that reported previously (IC_{50} = 8.1 mM) (Bränström et al., 1998). The patch-clamp experiments in our study were performed at room temperature, whereas insulin secretion was measured at 37°C. At 37°C, the curves in Fig. 4 might be slightly shifted to the right, because the K_d value for glibenclamide binding to the sulfonylurea receptors in membrane preparations of cerebral cortex (mainly SUR1) increased by 2.5-fold with the transition from room temperature to 37°C (temperature had no effect on the density of binding sites) (Gopalakrishnan et al., 1991). This finding and the lack of information on the cytosolic concentrations of α-keto acid

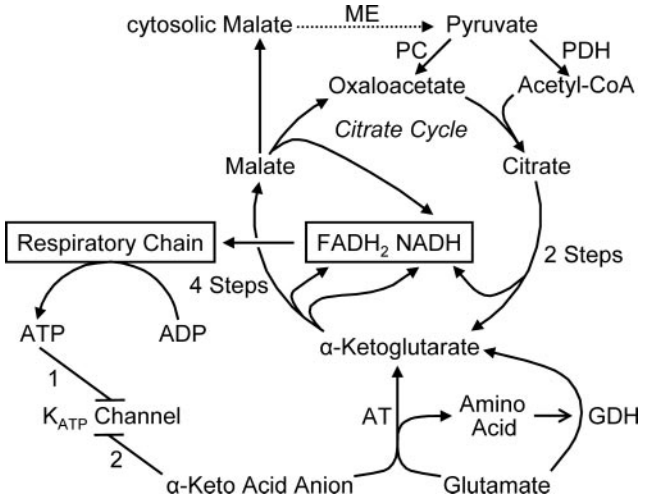

Fig. 7. Mechanism of the insulin-releasing action of α-keto acid anions (for references, see the text). α-Ketoisocaproate, α-ketocaproate, α-ketovalerate, α-ketoisovalerate, and β-phenylpyruvate are transaminated with L-glutamate by aminotransferases (AT) to form α-ketoglutarate and L-leucine, L-norleucine, L-norvaline, L-valine, and L-phenylalanine, respectively. L-Leucine, but not the other amino acids, strongly activates the glutamate dehydrogenase (GDH). α-Ketoglutarate and acetyl-CoA production enhance the citrate cycle activity and thereby the formation of NADH and $FADH_2$ (formation of GTP not shown). The resultant stimulation of the respiratory chain enhances the production of ATP. The increase in cytosolic ATP inhibits the K_{ATP} channel (1) (not shown: the simultaneous decrease in cytosolic ADP causes also K_{ATP} channel inhibition via a separate binding site at the channel). In addition, α-keto acid anions directly inhibit K_{ATP} channels (2). Complete intramitochondrial degradation provides the following products of one molecule of α-keto acid anion: 1 NADH + 1 $FADH_2$ + 1 acetoacetate + 1 acetyl-CoA + 1 CO_2 (consumption of 1 ATP) from α-ketoisocaproate; 2 NADH + 1 $FADH_2$ + 1 propionyl-CoA + 1 acetyl-CoA + 1 CO_2 from α-ketocaproate; 2 NADH + 1 $FADH_2$ + 2 acetyl-CoA + 1 CO_2 from α-ketovalerate; 3 NADH + 1 $FADH_2$ + 1 propionyl-CoA + 2 CO_2 from α-ketoisovalerate; degradation of β-phenylpyruvate in mouse β-cells is negligible. Only a small proportion of the acetoacetate produced from α-ketoisocaproate is oxidized in β-cells. Because the activity of malate enzyme (ME) is very low in mouse islets (MacDonald, 2002), malate formed by metabolism of propionyl-CoA does not provide acetyl-CoA in mouse islets (PDH, pyruvate dehydrogenase; PC, pyruvate carboxylase). The amounts of NADH and propionyl-CoA produced by degradation of α-ketoisovalerate are probably reduced by efflux of the metabolic intermediate 3-hydroxyisobutyrate. In addition to the initiation of insulin secretion by stimulation of ATP production, the metabolism of α-keto acid anions amplifies the initiated insulin secretion.

anions in mouse β-cells are the reasons why the data in Fig. 4 are not sufficient to decide whether direct K_{ATP} channel inhibition contributes to α-ketoisocaproate- and α-ketocaproate–induced insulin secretion.

Insulin secretion was amplified by α-keto acid anions in the order β-phenylpyruvate < α-ketoisovalerate < α-ketovalerate ≈ α-ketocaproate < α-ketoisocaproate (Fig. 6A). This order might reflect differences in metabolism of the α-keto acid anions (Fig. 7). The strong reduction of amplification by the transaminase inhibitor aminooxyacetate (Fig. 6B) is in favor of a major role of transaminations in α-keto acid anion-induced amplifications. The tested α-keto acid anions probably cause similar formation of α-ketoglutarate as a product of the transamination reactions (Malaisse et al., 1981; Lenzen et al., 1984, 1986). But differences in α-ketoglutarate formation seem to result from the supply of α-ketoglutarate by the glutamate dehydrogenase reaction in the β-cell mitochondria (Fig. 7). This reaction is strongly activated by L-leucine (Sener and Malaisse, 1980), which is produced by transamination of α-ketoisocaproate and has been proposed to contribute to the insulin-releasing effect of α-ketoisocaproate (Lenzen et al., 1986; MacDonald, 2002; Gao et al., 2003). L-Norvaline, the transamination product of α-ketovalerate, is a moderate activator of glutamate dehydrogenase (Lenzen et al., 1986). In contrast, L-norleucine, L-valine, and L-phenylalanine, the transamination products of α-ketocaproate, α-ketoisovalerate, and β-phenylpyruvate, respectively, are weak activators of glutamate dehydrogenase (Lenzen et al., 1986) and therefore probably do not promote α-ketoglutarate formation in β-cells. The strong activation of the glutamate dehydrogenase by L-leucine explains why α-ketoisocaproate amplifies insulin secretion much more than all of the other tested α-keto acid anions.

Both enhanced α-ketoglutarate production and acetyl-CoA formed by degradation of α-ketoisocaproate, α-ketocaproate, or α-ketovalerate activate the citrate cycle (Fig. 7). This acetyl-CoA formation might be the reason why not only α-ketoisocaproate but also α-ketocaproate and α-ketovalerate amplified insulin secretion much more than α-ketoisovale-rate and β-phenylpyruvate (Fig. 6A). In mouse islets, α-ketoisovalerate is decarboxylated at a high rate by the branched-chain keto acid dehydrogenase (Lenzen and Panten, 1980) but does not provide acetyl-CoA (MacDonald, 2002). Moreover, at an early step in the degradation of α-ketoisovalerate, 3-hydroxyisobutyrate is formed, substantial amounts of which probably leave the β-cells, as observed for other cell types (Corkey et al., 1982; Letto et al., 1990). Hence, α-ketoisovalerate is a moderate activator of the citrate cycle in mouse β-cells. Besides formation of CoA-ester intermediates, the degradation of α-ketoisocaproate, α-ketocaproate, α-ketovalerate, and α-ketoisovalerate supplies reducing equivalents (NADH and $FADH_2$) that can enhance the ATP production but do not explain the differences in amplification (Fig. 7). β-Phenylpyruvate is probably a weaker amplifier of insulin release than all other tested α-keto acid anions because its oxidation is insignificant in mouse islets (Lenzen and Panten, 1981).

In β-cells with active K_{ATP} channels, the potency of α-keto acid anions for initiation of insulin release increased in an order (Fig. 5A) different from that observed for amplification of insulin secretion (Fig. 6A). ATP-production by α-ketoisovalerate metabolism was apparently so low that no or only insignificant insulin release was caused in the absence of any other fuel or secretagogue (Fig. 5B) (Panten et al., 1972; Matschinsky et al., 1973; Lenzen and Panten, 1980). α-Ketovalerate produced first and second phases of insulin secretion which were much weaker than the corresponding responses to α-ketocaproate (Fig. 5A) (Lenzen, 1978). These findings cannot be explained by differences in metabolism of α-ketovalerate and α-ketocaproate, because the two compounds displayed similar amplification of insulin secretion. It is therefore concluded that the secretory response to α-ketocaproate, but not the response to α-ketovalerate, partially resulted from direct K_{ATP} channel inhibition. This view holds true also for α-ketoisocaproate, because the potencies of α-ketoisocaproate and α-ketocaproate for direct K_{ATP} channel inhibition were quite similar (Fig. 4). Although β-phenylpyruvate was the weakest amplifier of the tested α-keto acid anions, it triggered an initial peak of insulin release not lower than the corresponding peaks of the other α-keto acid anions. These results suggest that direct K_{ATP} channel inhibition (blocking ~70% of the K_{ATP} channels; see *Results*) is the major cause for initiation of insulin secretion by β-phenylpyruvate (10 mM). Because initiation of insulin secretion required closure of more than 98% of all K_{ATP} channels in β-cells (Panten et al., 1990), K_{ATP} channel inhibition by ATP (produced via β-phenylpyruvate transamination and enhanced oxidation of endogenous fuels) (Malaisse et al., 1983; Lenzen et al., 1984) probably contributed to β-phenylpyruvate-induced insulin secretion. A decrease in the latter contribution caused by consumption of endogenous fuels might explain why β-phenylpyruvate (10 mM) did not release insulin during the last 20 min of the test period (Fig. 5A), in contrast to 0.1 mM meglitinide (corresponding to a free meglitinide concentration blocking all K_{ATP} channels) (Panten et al., 1989; Schwanstecher et al., 1994) (Fig. 5B).

In conclusion, α-ketoisocaproate and related α-keto acid anions stimulate insulin secretion by acting as sulfonylurea analogs and/or by serving as substrates for transamination with glutamate or glutamine (Fig. 7). In β-cells, the sulfonylurea-like effect through interaction with SUR1 directly inhibits K_{ATP} channels, whereas transamination provides α-ketoglutarate, which indirectly inhibits K_{ATP} channels via activation of citrate cycle and mitochondrial ATP production. When the combined direct and indirect K_{ATP} channel inhibition is strong enough, insulin release is initiated. In addition, the increase in mitochondrial α-ketoglutarate and ATP production amplifies the initiated secretion. Differences in the insulin-releasing capacity of the individual α-keto acid anions result from differences in affinity to the sulfonylurea receptor, from differences in the production of extra α-ketoglutarate by activation of the glutamate dehydrogenase, and from differences in supply of acetyl-CoA.

Acknowledgments

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Address correspondence to: Dr. Uwe Panten, Institute of Pharmacology and Toxicology, Technical University of Braunschweig, Mendelssohnstrasse 1, D-38106 Braunschweig, Germany. E-mail: u.panten@tu-bs.de