Direct Inhibition of the Pancreatic β-Cell ATP-regulated Potassium Channel by α-Ketoisocaproate*

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The ATP-regulated potassium (K$_{ATP}$) channel plays an essential role in the control of insulin release from the pancreatic β-cell. In the present study we have used the patch-clamp technique to study the direct effects of α-ketoisocaproate on the K$_{ATP}$ channel in isolated patches and intact pancreatic β-cells. In excised inside-out patches, the activity of the K$_{ATP}$ channel was dose-dependently inhibited by α-ketoisocaproate, half-maximal concentration being approximately 8 mM. The blocking effect of α-ketoisocaproate was fully reversible. Stimulation of channel activity by the addition of ATP/ADP (ratio 1) did not counteract the inhibitory effect of α-ketoisocaproate. In the presence of the metabolic inhibitor sodium azide, α-ketoisocaproate was still able to inhibit single channel activity in excised patches and to block whole cell K$_{ATP}$ currents in intact cells. No effect of α-ketoisocaproate could be obtained on either the large or the small conductance Ca$^{2+}$-regulated K$^+$ channel. Enzymatic treatment of the patches with trypsin prevented the inhibitory effect of α-ketoisocaproate. Based on these observations, it is unlikely that the blocking effect of α-ketoisocaproate is due to an unselective effect on K$^+$ channel pores. Leucine, the precursor of α-ketoisocaproate, did not affect K$_{ATP}$ channel activity in excised patches. Our findings are compatible with the view that α-ketoisocaproate not only affects the β-cell stimulus-secretion coupling by generation of ATP but also by direct inhibition of the K$_{ATP}$ channel.

An increase in plasma glucose concentration is the major physiological stimulus for insulin release from the pancreatic β-cell. Glucose metabolism leads to membrane depolarization and initiation of a characteristic pattern in electrical activity concomitant with fluctuations in cytosolic-free Ca$^{2+}$-concentration (1). Depolarization results from an increase in the ATP/ADP ratio, thereby inducing a closure of the ATP-dependent K$^+$ (K$_{ATP}$) channel (2). An increase in mitochondrial metabolism plays an important role in the response to glucose and several other fuel secretagogues. Among other fuel secretagogues, the deamination product of the amino acid L-leucine, α-ketoisocaproate, is of particular interest since it is exclusively metabolized in mitochondria (3, 4). Accordingly, several studies have shown that α-ketoisocaproate stimulates insulin secretion (3, 4), initiates extracellular activity (5), and inhibits the β-cell K$_{ATP}$ channel in intact cells monitored in the cell-attached configuration of the patch-clamp technique (6–8). The fact that metabolism of α-ketoisocaproate is confined to the mitochondria indicates that a product in Krebs cycle is involved in modulation of channel activity. The most likely candidate is ATP, which has been shown to be elevated after exposure of β-cells to α-ketoisocaproate (4, 6). In the present study we have further investigated the effects of α-ketoisocaproate on the K$_{ATP}$ channel and demonstrate that the substance directly inhibits the β-cell K$_{ATP}$ channel.

EXPERIMENTAL PROCEDURES

Preparation—Adult obese mice (gene ob/ob) of both sexes were obtained from a local colony (9). The mice were starved for 24 h and then killed by decapitation. Pancreatic islets were isolated by a collagenase technique (10), and a cell suspension was prepared and washed essentially as described previously (11). The cells were resuspended in RPMI 1640 culture medium (Flow Laboratories, Scotland, UK) containing 11 mM glucose supplemented with 10% fetal bovine serum, 100 IU/ml penicillin, 100 μg/ml streptomycin, and 60 μg/ml gentamycin. Collagenase was obtained from Boehringer Mannheim. The cell suspension was seeded into Petri dishes (Corning Glass, Corning, NY) and incubated at 37 °C in 5% CO$_2$ for 1–3 days.

Solutions—The bath solution (i.e. the “intracellular” solution) consisted of 125 mM KCl, 1 mM MgCl$_2$, 10 mM EGTA, 30 mM KOH, and 5 mM HEPES-NaOH (pH 7.15) unless otherwise indicated. In the experiments using the inside-out configuration, the pipettes were filled with standard extracellular solution containing 138 mM NaCl, 5.6 mM KCl, 1.2 mM Mg$_2$Cl$_2$, 2.6 mM CaCl$_2$, and 5 mM HEPES-NaOH (pH 7.40). In the perforated patch experiments, the pipette solution contained 10 mM KCl, 76 mM K$_2$SO$_4$, 10 mM NaCl, 1 mM MgCl$_2$, and 10 mM NaOH (pH 7.15), and 200 μg of amphotericin B/ml (dissolved in Me$_2$SO). The final concentration of Me$_2$SO was less than 0.1%. ATP and ADP (both supplied by Sigma) were added to the intracellular solution as indicated. When nucleotides were added as their Na$^+$ salt (ADP), Mg$^{2+}$ was added to maintain an excess of Mg$^{2+}$. α-Ketoisocaproate-Na$^+$ salt was obtained from two different suppliers (Sigma and Fluka Chemie AG, Neu-Ulm, Switzerland), and 2-oxopentanoate was obtained from Aldrich. Trypsin-EDTA was purchased from Life Technologies, Inc.

Electrophysiology—K$_{ATP}$ channel activity and membrane potential were recorded using the patch-clamp technique (12). Pipettes were coated with Sylgard resin (Dow Corning, Kanagawa, Japan) near their tips to reduce capacitance transients and, finally, were fire-polished. Currents were recorded using an Axopatch 200 patch-clamp amplifier (Axon Instruments, Inc., Foster City, CA). Experiments were stored on magnetic tape using a video cassette recorder (JVC, Tokyo, Japan) and a digital data recorder (VR-10B, Instrutech Corp., Elmont, NY). The recorded signal was stored with an upper cut-off frequency of 2 kHz. Patches were excised into a nucleotide-free solution as indicated. When nucleotides were added as their Na$^+$ salt, 0.1 mM ATP was first added to test for channel inhibition. With the solutions used, ion currents were outward, and channel records were displayed according to the convention; upward deflections denote outward currents. All experiments were performed at room temperature (20–24 °C), and channel activity was measured at 0 mV unless otherwise indicated. Whole cell K$_{ATP}$ currents and β-cell membrane potential were recorded using the perforated patch configuration of the patch-
clamping technique.

For analysis of single channel kinetics, records were low-pass filtered at 0.2 kHz using an 8-pole Bessel filter (Frequency Devices, Haverhill, MA) digitized at 0.8 kHz using a TL-1 DMA interface (Axon Instruments) and stored in a computer. Open time kinetics were determined using in-house software by digitizing segments of the current records (~60 s long) and forming histograms of base-line and open-level data points. Analysis of the distribution of $K_{ATP}$ channel open times was restricted to segments containing no more than three active channels. Events were identified using a 50% amplitude criterion. The kinetic constants were derived by approximation of the data to exponential functions by the method of maximum likelihood (13). Channel activity constants were derived by approximation of the data to exponential events were identified using a 50% amplitude criterion. The kinetic constants were derived by approximation of the data to exponential functions by the method of maximum likelihood (13). Channel activity constants were derived by approximation of the data to exponential functions by the method of maximum likelihood (13).

The $K_{ATP}$ Channel Activity Inhibited by $\alpha$-Ketoisocaproate—In the intact $\beta$-cell, the activity of the $K_{ATP}$ channel is the main regulator of resting membrane potential, and the intracellular ATP/ADP ratio is considered to constitute the primary determinant of channel activity (14). It is obvious that substances that are capable of modulating channel activity may also influence resting membrane potential. Fig. 1A shows a typical trace exposing an excited patch to 20 mM $\alpha$-ketoisocaproate. When exposing patches to 20 mM $\alpha$-ketoisocaproate, channel activity ($NP_b$) decreased to 31% ± 5% ($n = 6; p < 0.001$) compared with what was found before the addition of the substance. In Fig. 1B, we exposed isolated patches to an ATP/ADP ratio of 1, which efficiently stimulates $K_{ATP}$ channel activity (15). Upon the addition of the nucleotides, mean current increased with 247% ± 74% ($n = 3$) and induced a typical kinetic pattern with openings of long duration (16). The addition of $\alpha$-ketoisocaproate (20 mM) to the patch in the continued presence of nucleotides reduced channel activity to 26% ± 7% ($n = 4; p < 0.05$). These data clearly show that the $K_{ATP}$ channel can be directly modulated by $\alpha$-ketoisocaproate in inside-out patches.

Since $\alpha$-ketoisocaproate is a Na$^+$ salt, the decrease in single channel unitary conductance from 19.1 ± 0.3 picoSiemens to 14.3 ± 0.4 picoSiemens ($n = 7; p < 0.01$) after the addition of this fuel secretagogue could be explained by the presence of Na$^+$ alone. In the millimolar range, Na$^+$ is known to interact with channel conductance (17, 18). Thus, the obtained decreases in channel conductance by the various concentrations of the $\alpha$-ketoisocaproate-Na$^+$ salt were identical to those reported earlier by Na$^+$ (17). When correcting for the Na$^+$ concentration during and before exposure of the patches to $\alpha$-ketoisocaproate, no alteration in unitary conductance was observed (Fig. 1C).

In a series of experiments we exposed patches to four different concentrations of $\alpha$-ketoisocaproate ranging from 2 to 20 mM. It is clear that $\alpha$-ketoisocaproate dose-dependently decreased $K_{ATP}$ channel activity (Fig. 2A). Compiled data of the concentration-inhibition relation is shown in Fig. 2B. Values are expressed as the ratio of channel activity obtained in the presence of $\alpha$-ketoisocaproate ($NP_b$) and the activity assessed in the standard intracellular solution before the addition of $\alpha$-ketoisocaproate ($NP_p$ Control). The mean value points were fitted to the Hill equation. Estimates of the concentration causing a 50% reduction in channel activity was found to be 8.1 mM, with a Hill coefficient of 2.3, which suggests that the block involves the binding of more than one molecule to the $K_{ATP}$ channel complex.

We further examined the effects of $\alpha$-ketoisocaproate on kinetic properties of the $K_{ATP}$ channel. In Fig. 2C we analyzed the open-time distribution in the presence of $\alpha$-ketoisocaproate using patches containing no more than three simultaneously active channels. Insets show examples of channel openings under the respective experimental conditions on an expanded time scale. The mean duration of openings under control conditions was 34.1 ± 1.1 ms ($n = 5$), which is similar to what has been reported earlier for the $K_{ATP}$ channel (16). The same type of channel activity was observed in the presence of $\alpha$-ketoisocaproate, with a mean open time of 29.7 ± 2.1 ms ($n = 4$; n.s.).

The Inhibitory Effects of $\alpha$-Ketoisocaproate on the $K_{ATP}$ Channel Is Not Dependent on Metabolism—To further verify that $\alpha$-ketoisocaproate has the ability to directly inhibit the $K_{ATP}$ channel without involving metabolism of the substance, we investigated $K_{ATP}$ channel activity in intact cells in the presence of the metabolic inhibitor sodium azide (NaN$_3$) using the perforated patch configuration of the patch-clamp technique. It is well established that the input conductance in the unstimulated $\beta$-cell is dominated by the $K_{ATP}$ channel and this conductance is virtually completely inhibited by the sulfonylurea com-

![Fig. 1. The effects of $\alpha$-ketoisocaproate on $K_{ATP}$ channel activity in inside-out patches from pancreatic $\beta$-cells.](image-url)
stimulation with reported that the onset of insulin secretion is more rapid after a decrease in channel activity from 2.06 to 0.98, and finally, exposing the patch to 5 mM glucose (4). Finally, we studied the effect of NaN$_3$ in the presence of 20 mM glucose (4). The data were fitted to the Hill equation. Results are presented as mean values ± S.E.M. for 4–6 observations. C, frequency versus lifetime histograms of channel openings under control condition and in the presence of α-ketoisocaprate. In control solution, the distribution of channel open time could be described by a single exponential function with a time constant ($\tau$) of 37.6 ms. A total of 856 events were analyzed. In the presence of 20 mM α-ketoisocaprate, the distribution of open time could be fitted with a $\tau$ of 33.1 ms (978 events). The insets show typical channel activity on an expanded time scale. Arrowheads indicate the current level when the channel is closed. A sample frequency of 0.8 kHz was used, and the bin width was set to 25 ms.

FIG. 2 Dose-response and kinetic effects of α-ketoisocaprate on $K_{ATP}$ channel activity in inside-out patches. A, typical recording of $K_{ATP}$ currents in the presence of different concentrations of α-ketoisocaprate. The data were fitted to the Hill equation. Results are presented as mean values ± S.E.M. for 4–6 observations. C, frequency versus lifetime histograms of channel openings under control condition and in the presence of α-ketoisocaprate. In control solution, the distribution of channel open time could be described by a single exponential function with a time constant ($\tau$) of 37.6 ms. A total of 856 events were analyzed. In the presence of 20 mM α-ketoisocaprate, the distribution of open time could be fitted with a $\tau$ of 33.1 ms (978 events). The insets show typical channel activity on an expanded time scale. Arrowheads indicate the current level when the channel is closed. A sample frequency of 0.8 kHz was used, and the bin width was set to 25 ms.

We have previously shown that glucose-induced membrane depolarization and increase in cytosolic Ca$^{2+}$ concentration are slower at room temperature compared with 37 °C (19), most likely reflecting a lower metabolic rate in the cell. We therefore estimated the time from the addition of α-ketoisocaprate or 15 mM glucose to the appearance of action potentials at high (34 °C) and low (26 °C) temperature. Top traces in Fig. 3C show recordings of membrane potential after the addition of 20 mM α-ketoisocaprate at low and high temperature. No significant change in the latency between the addition and appearance of action potential was observed using α-ketoisocaprate, 11.2 ± 0.9 s versus 9.8 ± 1.6 s at low and high temperature, respectively (n = 4; n.s.). In contrast, when adding 15 mM glucose (bottom traces), a significant delay was observed when lowering the temperature to 26 °C, 37.7 ± 6.7 s at 34 °C 86.2 ± 20.5 s at 26 °C (n = 6; p < 0.05). It should be pointed out that it has been reported that the onset of insulin secretion is more rapid after stimulation with α-ketoisocaprate compared with stimulation with glucose (4). Finally, we studied the effect of NaN$_3$ in inside-out patches. As seen in Fig. 3D, channel activity remained unchanged after the addition of NaN$_3$ to the bath. In the presence of NaN$_3$, α-ketoisocaprate (bottom trace) still potently inhibited channel activity. Taken together, these data strongly suggest that α-ketoisocaprate can block $K_{ATP}$ channel activity independent of metabolism of the keto acid.

Effects on $K_{ATP}$ Channel Activity by l-Leucine and 2-Oxopentanoate—Several studies have described insulinotropic effects of l-leucine (3, 20, 21). Because of the blocking effect of α-ketoisocaprate on the $K_{ATP}$ channel, we also investigated whether the precursor amino acid, l-leucine, had direct effects on the channel in excised patches. As shown in Fig. 4A, the addition of 20 mM l-leucine to an isolated patch neither affected single $K_{ATP}$ channel activity nor channel mean open time (30.4 ± 2.8 ms; n = 4; n.s.). Subsequent inclusion of 20 mM α-ketoisocaprate in the same patch significantly inhibited channel activity. l-Arginine, which has a close structural resemblance to l-leucine, was also without effect on $K_{ATP}$ channel activity (data not shown). Oxopentanoate, which is derived from pentanoic acid, differs from α-ketoisocaprate in lacking a methyl group. Like α-ketoisocaprate, 2-oxopentanoate potently inhibited $K_{ATP}$ channel currents (Fig. 4B).

Specific Effect on the $K_{ATP}$ Channel—In contrast to the potent inhibition of the $K_{ATP}$ channel by α-ketoisocaprate, no effect could be seen on the large conductance Ca$^{2+}$-regulated K$^+$ ($K_{BKC}$) channel (14). As seen in Fig. 5A, exposing inside-out patches to 20 mM α-ketoisocaprate, no effect on the mean current of $K_{BKC}$ channel could be monitored. In a series of experiments, $K_{BKC}$ channel activity (NP$_6$) was assessed to
FIG. 3. Effects of NaN₃ on α-ketoisocaproate-induced inhibition of Kₐtp channel activity in intact cells (A-C) using the perforated-patch configuration and in excised inside-out patches (D). A, cells were voltage-clamped (V-C) at a holding potential of −70 mV, and voltage excursions of ±5 mV (200 ms) were performed (top left). In the presence of 1 mM NaN₃, input conductance increased more than 5-fold, from 0.9 nS to 5.2 nS (bottom left). The addition of 15 mM glucose in the presence of NaN₃ did not affect the Kₐtp currents. In the presence of NaN₃, input conductance was estimated to 3.0 nS and 3.1 nS before and after the addition of glucose, respectively (top right). Bottom right shows currents in the presence and absence of α-ketoisocaproate in the continued presence of sodium azide. The addition of the keto acid decreased the conductance from 5.3 nS to 2.5 nS. B, compiled data on the effects on input conductance after the addition of 15 mM glucose and 20 mM α-ketoisocaproate in the presence of 1 mM NaN₃. The recordings were made at 34 °C, and the effect of glucose was estimated after ≥120 s, which should be sufficient time for the fuel to act. C, effects of temperature on membrane potential when stimulated with 20 mM α-ketoisocaproate (top panels). At low temperature (26 °C; left), the addition of the keto acid caused the cell to depolarize and display continues action potentials. The time from the addition of α-ketoisocaproate to the appearance of action potentials was estimated to 14.5 s. The time was not significantly affected by increasing the temperature to 34 °C (13.2 s; right). Lower panels, at 26 °C, the time span from the addition of 15 mM glucose to the appearance of action potentials was 55.1 s (left). Elevating the temperature to 34 °C resulted in a shortening of the time to 31.5 s (right). D, 1 mM NaN₃ did not affect Kₐtp channel currents in inside-out patches (top trace); NPO before azide was estimated to 3.72 and after addition of azide to 3.45. The small decrease in channel activity could well be accounted for by spontaneous channel rundown. ** p < 0.01, compared with NaN₃ alone.

FIG. 4. Effects on Kₐtp channel activity by L-leucine and 2-oxopentanoate. A, inclusion of L-leucine (20 mM) in the intracellular solution did not affect Kₐtp channel activity. NPO was estimated to 2.39 under control conditions and 2.31 in the presence of the amino acid, whereas the addition of 20 mM α-ketoisocaproate to the same patch reduced channel activity to 0.57. B, the addition of 2-oxopentanoate reduced channel activity from 7.05 to 0.89. The structures of L-leucine, 4-methyl-2-oxopentanoic acid (α-ketoisocaproate), and 2-oxopentanoate are shown in C. The vertical calibration bar represents 5 pA in A and 10 pA in B. The above experiments were repeated at least four times with similar results.
ATP-dependent K⁺ Channel Inhibition by α-Ketoisocaprate

Effect of ATP remains, although with slightly decreased efficiency (22, 23). Evidently this action of trypsin treatment results from its specific proteolytic effects on the K_ATP channel complex (22), trypsin having a primary affinity for arginine and lysine residues (24). After trypsin modification, inclusion of α-ketoisocaprate (20 mM) in the bath solution was unable to affect K_ATP channel activity (Fig. 5C). This experimental protocol was repeated four times, and in all four patches the inhibitory effect of α-ketoisocaprate on K_ATP channel activity was lost subsequent to trypsin treatment (Fig. 5D). Channel activity (N_Pₜₜ) was 3.1 ± 0.5 and 3.0 ± 0.5 (n = 4; n.s.) before and after the addition of α-ketoisocaprate, respectively. Based on these observations, it is highly unlikely that the effect of α-ketoisocaprate is due to a nonspecific block of any K⁺ channel pore but rather involves a specific interaction with the K_ATP channel protein complex.

Concluding Remarks—It has been known for the last decade that the deamination product of L-leucine, α-ketoisocaprate, is a potent stimulator of insulin secretion (21). A number of studies have demonstrated that this compound, like glucose, causes inhibition of the K_ATP channel in the pancreatic β-cell (6–8, 21). In addition, blocking of the respiratory cycle reduces the insulinotrophic effect of α-ketoisocaprate (4). These observations have suggested a role for oxidative phosphorylation-generated messengers to promote closure of the K_ATP channel. In contrast to these findings, the results presented in this study clearly show that α-ketoisocaprate directly and reversibly inhibits the K_ATP channel in the pancreatic β-cell without involving metabolism of the substance. Since the resting potential in the intact β-cell is mainly determined by the activity of the K_ATP channel (14), it is clear that α-ketoisocaprate will also influence membrane potential and thereby insulin secretion by direct inhibition of the channel. Thus, in view of our findings, the use of α-ketoisocaprate as a tool to study the role of mitochondrial metabolism in intact β-cells should be interpreted cautiously. To what extent direct effects of α-keto acids on K_ATP channel activity are involved in the β-cell stimulus-secretion coupling is at present difficult to assess. In our view, the concentrations required to affect the activity of the K_ATP channel, as found in the present study, are not likely to be reached during physiological conditions.

The present design does not allow us to evaluate relative contribution of the direct effect of α-ketoisocaprate on the K_ATP channel versus effects resulting from metabolism of the substance. In this context it is interesting that α-ketoisocaprate stimulates insulin release more rapidly than glucose (4). The direct effect of α-ketoisocaprate on the K_ATP channel may explain this difference in kinetics in the stimulation of insulin release.

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**Fig. 5. Specific effect on the K_ATP channel.** A, to record the Ca²⁺-activated large conductance Kᵦₛ channel, EGTA was omitted and 100 μM CaCl₂ was added to the bath solution. Currents were recorded at a membrane potential of 0 mV. Typical recording showing that the open probability of Kᵦₛ channel was unchanged during exposure to 20 mM α-ketoisocaprate. This experimental protocol was repeated three times with identical results. B, amplitude histograms from the trace in A in the absence and presence of 20 mM α-ketoisocaprate. C, the top trace shows a typical recording of single Kᵦₛ channel currents after an approximately 5-min exposure of the patch to 20 μg/ml trypsin. Channel activity was unchanged after exposure of the patch to 20 mM α-ketoisocaprate. D, compiled data of the effect of 20 mM α-ketoisocaprate from nine patches before (left) and after (right) trypsin. Each symbol represents a different patch. C denotes channel closed.

0.61 ± 0.09 during control solution, compared with 0.60 ± 0.10 in the presence of α-ketoisocaprate (n = 3; n.s.). The activity of the small Ca²⁺-regulated K⁺ conductance channel (Kᵦᵢ) of pancreatic β-cells (14) was also unaffected after inclusion of 20 mM α-ketoisocaprate in the bath solution (data not shown).

To further investigate whether the effect of α-ketoisocaprate is specific for the K_ATP channel, we studied the effects of α-ketoisocaprate on channel activity in trypsin-modified patches. Exposure of isolated membrane patches to 20 μg/ml trypsin for ~5 min has been reported to alter K_ATP channel activity with a specific pattern. Thus, trypsin treatment takes away the activating effect of MgADP and the inhibitory effect of sulfonylureas on K_ATP channel activity, whereas the inhibitory effect of ATP remains, although with slightly decreased efficiency (22, 23). Evidently this action of trypsin treatment results from its specific proteolytic effects on the K_ATP channel complex (22), trypsin having a primary affinity for arginine and lysine residues (24). After trypsin modification, inclusion of α-ketoisocaprate (20 mM) in the bath solution was unable to affect K_ATP channel activity (Fig. 5C). This experimental protocol was repeated four times, and in all four patches the inhibitory effect of α-ketoisocaprate on K_ATP channel activity was lost subsequent to trypsin treatment (Fig. 5D). Channel activity (N_Pₜₜ) was 3.1 ± 0.5 and 3.0 ± 0.5 (n = 4; n.s.) before and after the addition of α-ketoisocaprate, respectively. Based on these observations, it is highly unlikely that the effect of α-ketoisocaprate is due to a nonspecific block of any K⁺ channel pore but rather involves a specific interaction with the K_ATP channel protein complex.

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