Mol 15388

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.)

1

Mol 15388

Running title: Insulin-releasing action of α-ketoisocaproate

Corresponding author: Uwe Panten

Institute of Pharmacology and Toxicology

Technical University of Braunschweig

Mendelssohnstrasse 1

D-38106 Braunschweig

Germany

Tel.: +49-531-3915669

Fax: +49-531-3918182

E-mail: u.panten@tu-bs.de

Text pages: 24

Tables: 1

Figures: 7

References: 42

Words in the Abstract: 249

Words in the Introduction: 544

Words in the Discussion: 1553

Nonstandard abbreviations: K_{ATP} channel, ATP-sensitive K^+ channel; SUR1, sulfonylurea receptor 1; SUR2B, sulfonylurea receptor 2B; K_{IR}, potassium inward rectifier channel.

Mol 15388

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 (SUR1), 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.

Introduction

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 rise 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, 2-oxohexanoate; α-ketovalerate, 2-oxopentanoate; β-phenylpyruvate, 2-oxo-3-phenylpropionate) stimulate insulin secretion by pancreatic islets in the absence of any other fuel or secretagogue (Panten et al., 1972; Matschinsky et al., 1973; Lenzen and Panten, 1980; Hutton et al., 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; Malaisse et al., 1983; Lenzen et al., 1984; Lenzen et al., 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; 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 since 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 labelled 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) and of EGFP (enhanced green fluorescent protein). 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 48-72 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 sexes (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 (presence of 10 mM glucose) on 35 mm dishes as detailed previously (Schwanstecher et al., 1994). Single channel currents were studied after 24-72 h of culture.

A standard patch-clamp technique was used in the inside-out configuration as previously described 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 (in mM) KCl 146, CaCl$_2$ 2.6, MgCl$_2$ 1.2,

and HEPES 10 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 (in mM) KCl 140, MgCl$_2$ 1, EGTA 10, CaCl$_2$ 2, and HEPES 5 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 appropriate

amounts of MgCl$_2$ (calculated as described by Schwanstecher et al., 1994). The bath was

perfused at 2 ml/min, and about 30 s were 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, Foster City, CA, USA) and analysed with the pCLAMP 6.0 software

(Axon Instruments).

For experiments with COS-7 cells channel activity (N · P$_O$) was calculated as:

$$(1) \quad N \cdot P_O = 1/T \cdot \Sigma n_i \cdot t_i$$

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 20-30 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. Firstly, islet cell suspensions prepared from mouse islets by the method also applied in our study contained only 2 or 5 % of δ- or PP-cells, respectively (Barg et al., 2000). Secondly, 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 around 1 or 20-30 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 complete closure of K_{ATP} channels by sulfonylureas and analogues, thereby facilitated analysis of concentration-response relationships, and avoided 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 base line and the activity of a channel with a lower single channel current amplitude (about 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 appear to be altered by ATP or the applied test compounds. All α-keto acids were applied as sodium salts since 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 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. Due to 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 ($N \cdot P_O$) according to

the equation

$$(2) \quad I = N \cdot P_O \cdot i$$

where i is the mean single K_{ATP} channel current amplitude (3.6 pA). Normalized channel

current was calculated as:

$$(3) \quad \text{Channel current (\%)} =$$

$$(I_{test}-I_{ATP}) \cdot 100/(I_{control}-I_{ATP})$$

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 60-90 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 detailed in Results) as described previously (Panten et al., 1989).

Supplementation of medium with glipizide or meglitinide was performed by 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 1-4 min fractions

was determined by enzyme-linked immunosorbent assay (ELISA kit, 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 Tris-HCl-buffer pH 7.4 (185 mM Tris) containing (final concentrations) [^{3}H]glibenclamide (0.3 nM) and test substances (final concentrations indicated in 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 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 in 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 $K_{IR}6.2\Delta C26$ 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 analogues (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 carboxy terminus of $K_{IR}6.2$ by the terminal 26 amino acids ($K_{IR}6.2\Delta C26$) 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 $K_{IR}6.2\Delta C26$ 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 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 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 60-100 mM concentrations of β-phenylpyruvate inhibited specific [³H]ligand binding by about 90 % (Fig. 2). Therefore relations between the concentrations of carboxylic acid anions and specific binding were analysed 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 [³H]ligand binding to SUR1 and inhibited [³H]ligand binding to SUR2B by about 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 due to the fact that no correction has been made for non-specific effects on SUR2B binding as revealed by high NaCl concentrations (see above).

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 (around 1.2 pA) were observed (Fig. 3). The current traces revealed pronounced run-down of K_{ATP} channel activity. Similar run-down occurred in nearly all experiments and was considered by including control periods before and after the test period (for calculation see Experimental procedures). 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 effects of undissociated n-hexanoic acid on the lipid phase of the patch membrane. 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 analysed 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 insulin/min per islet (n = 56, all experiments in

Fig. 5). In groups of 6-10 experiments the secretion rates at the end of the control period varied considerably due to 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 min 62.5 (Fig. 5A and B). Whereas the secretory rates at min 62.5 were similar for α-ketoisocaproate, α-ketocaproate, and β-phenylpyruvate, the rate for α-ketovalerate was significantly lower ($p < 0.02$, comparison with α-ketocaproate; $p < 0.05$, comparison with α-ketoisocaproate or β-phenylpyruvate). α-Ketoisovalerate caused only a very small peak of insulin secretion ($p < 0.02$, comparison with the rate at min 62.5 in the absence of test compound) (Fig. 5B). The tested α-keto acid anions (10 mM) differed also in the secretory profile following the initial peak of insulin release (Fig. 5A and B). Some compounds produced a second phase of insulin secretion which was strong and sustained (α-ketoisocaproate), strong, but gradually decreasing from min 78 to min 102 (α-ketocaproate), or weak (α-ketovalerate). β-Phenylpyruvate did not induce a second phase of insulin release. From min 82 to min 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 analogue meglitinide (0.1 mM) slightly enhanced the secretory rate at min 62.5 ($p < 0.02$) and induced a prolonged insulin release (average secretory rate from min 82 to min 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 perifusion experiments with islets exposed to the sulfonylurea glipizide at a concentration (2.7 μM) completely inhibiting the K_{ATP} channels of

14

β-cells (Panten et al., 1989). Complete 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 absence of any substrate, the insulin secretion rate was 14.4 ± 1.2 pg insulin/min per 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 ± 0.2 pg insulin/min per islet, see above). All tested α-keto acid anions (10 mM) significantly enhanced insulin secretion within 3-4 min (Fig. 6A). Secretory maxima were reached after different periods (14 min for α-ketoisocaproate and α-ketocaproate, 3-4 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). But 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 ≈ α-ketocaproate < α-ketoisocaproate, and the following significances were calculated for comparisons of total insulin release: β-phenylpyruvate vs α-ketoisovalerate, $p < 0.05$; α-ketoisovalerate vs α-ketovalerate, $p < 0.02$; α-ketovalerate vs α-ketocaproate, $p > 0.05$; α-ketocaproate vs 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).

Since 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 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 insulin/min per 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. 6A 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 analogues (Figs. 1-4, Table 1). As in the case of sulfonylureas and their analogues (Panten et al., 1989; Schwanstecher et al., 1994; Meyer et al., 1999), Hill coefficients for binding were close to 1, 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 analogue meglitinide (Meyer et al., 1999): an α-keto group in the molecules was not essential for 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).

16

The receptor site for sulfonylureas and their analogues 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; Malaisse et al., 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 about 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 3-4 mM in the β-cell cytosol, assuming restriction of the α-keto acid anions to the cytosol and the mitochondrial matrix (Dean, 1973). At 3-4 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 3-4 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 analogues 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 run-down (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; Bränstöm et al.,

17

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, since 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 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 favour 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; Lenzen et al., 1986). But differences in α-ketoglutarate formation seem to result from 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 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-ketoisovalerate 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 $FADH_2$) which 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 since 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 a minute 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 since 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). Since 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 analogues 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 production of extra α-ketoglutarate by activation of the glutamate dehydrogenase, and from differences in supply of acetyl-CoA.

References

Aguilar-Bryan L and Bryan J (1999) Molecular biology of adenosine triphosphate-sensitive potassium channels. *Endocr Rev* 20:101-135.

Ashcroft FM, Ashcroft SJH and Harrison DE (1987) Effects of 2-ketoisocaproate on insulin release and single potassium channel activity in dispersed rat pancreatic β-cells. *J Physiol* 385:517-529.

Ashfield R, Gribble FM, Ashcroft SJ and Ashcroft FM (1999) Identification of the high-affinity tolbutamide site on the SUR1 subunit of the K_{ATP}-channel. *Diabetes* 48:1341-1347.

Barg S, Galvanovskis J, Göpel SO, Rorsman P and Eliasson L (2000) Tight coupling between electrical activity and exocytosis in mouse glucagon-secreting α-cells. *Diabetes* 49:1500-1510.

Bränström R, Efendić S, Berggren P-O and Larsson O (1998) Direct inhibition of the pancreatic β-cell ATP-regulated potassium channel by α-ketoisocaproate *J Biol Chem* 273:14113-14118.

Cheng Y-C and Prusoff WH (1973) Relationship between the inhibition constant (K_i) and the concentration of inhibitor which causes 50 per cent inhibition (I_{50}) of an enzymatic reaction. *Biochem Pharmacol* 22:3099-3108.

Corkey BE, Martin-Requero A, Walajtys-Rode E, Williams RJ and Williamson JR (1982) Regulation of the branched chain α-ketoacid pathway in liver. *J Biol Chem* 257:9668-9676.

Dean PM (1973) Ultrastructural morphometry of the pancreatic β-cell. *Diabetologia* 9:115-119.

Dörschner H, Brekardin E, Uhde I, Schwanstecher C and Schwanstecher M (1999)
Stoichiometry of sulfonylurea-induced ATP-sensitive potassium channel closure. *Mol Pharmacol* 55:1060-1066.

Duchen MR, Smith PA and Ashcroft FM (1993) Substrate-dependent changes in mitochondrial function, intracellular free calcium concentration and membrane channels in pancreatic β-cells. *Biochem J* 294:35-42.

Gao Z, Young RA, Li G, Najafi H, Buettger C, Sukumvanich SS, Wong RK, Wolf BA and Matschinsky FM (2003) Distinguishing features of leucine and α-ketoisocaproate sensing in pancreatic β-cells. *Endocrinology* 144:1949-1957.

Gopalakrishnan M, Johnson DE, Janis RA and Triggle DJ (1991) Characterization of binding of the ATP-sensitive potassium channel ligand, [^{3}H]glyburide, to neuronal and muscle preparations. *J Pharmacol Exp Ther* 257:1162-1171.

Gribble FM, Tucker SJ and Ashcroft FM (1997) The interaction of nucleotides with the tolbutamide block of cloned ATP-sensitive K^+ channel currents expressed in Xenopus oocytes: a reinterpretation. *J Physiol* 504:35-45.

Henquin J-C (2000) Triggering and amplifying pathways of regulation of insulin secretion by glucose. *Diabetes* 49:1751-1760.

Hutson SM, Roten S and Kaplan RS (1990) Solubilization and functional reconstitution of the branched-chain α-keto acid transporter from rat heart mitochondria. *Proc Natl Acad Sci USA* 87:1028-1031.

Hutton JC, Sener A and Malaisse WJ (1979) The metabolism of 4-methyl-2-oxopentanoate in rat pancreatic islet. *Biochem J* 184:291-301.

Hutton JC, Sener A, Herchuelz A, Atwater I, Kawazu S, Boschero C, Somers G, Devis G and Malaisse WJ (1980) Similarities in the stimulus-secretion coupling mechanisms of glucose- and 2-keto acid-induced insulin release. *Endocrinology* 106:203-219.

Lenzen S (1978) Effects of α-ketocarboxylic acids and 4-pentenoic acid on insulin secretion from the perfused rat pancreas. *Biochem Pharmacol* 27:1321-1324.

Lenzen S and Panten U (1980) 2-Oxocarboxylic acids and function of pancreatic islets in obese-hyperglycaemic mice. Insulin secretion in relation to ^{45}Ca uptake and metabolism. *Biochem J* 186:135-144.

Lenzen S and Panten U (1981) Effects of pyruvate, L-lactate, and 3-phenylpyruvate on function of ob/ob mouse pancreatic islets: insulin secretion in relation to ^{45}Ca^{2+} uptake and metabolism. *Biochem Med* 25:366-372.

Lenzen S, Rustenbeck I and Panten U (1984) Transamination of 3-phenylpyruvate in pancreatic B-cell mitochondria. *J Biol Chem* 259:2043-2046.

Lenzen S, Schmidt W, Rustenbeck I and Panten U (1986) 2-Ketoglutarate generation in pancreatic B-cell mitochondria regulates insulin secretory action of amino acids and 2-keto acids. *Biosci Rep* 6:163-169.

Lernmark Å (1974) The preparation of, and studies on, free cell suspensions from mouse pancreatic islets. *Diabetologia* 10:431-438.

Letto J, Brosnan JT and Brosnan ME (1990) Oxidation of 2-oxoisocaproate and 2-oxoisovalerate by the perfused rat heart. Interactions with fatty acid oxidation. *Biochem Cell Biol* 68:260-265.

MacDonald MJ (2002) Differences between mouse and rat pancreatic islets: succinate responsiveness, malic enzyme, and anaplerosis. *Am J Physiol Endocrinol Metab* 283:E302-E310.

MacDonald MJ, Fahien LA, Brown LJ, Hasan NM, Buss JD and Kendrick MA (2005) Perspective: emerging evidence for signaling roles of mitochondrial anaplerotic products in insulin secretion. *Am J Physiol Endocrinol Metab* 288:E1-E15.

Malaisse WJ, Sener A, Malaisse-Lagae F, Hutton JC and Christophe J (1981) The stimulus-secretion coupling of amino acid-induced insulin release. Metabolic interaction of glutamine and 2-ketoisocaproate in pancreatic islets. *Biochim Biophys Acta* 677:39-49.

Malaisse WJ, Malaisse-Lagae F and Sener A (1982) The stimulus-secretion coupling of glucose-induced insulin release: effect of aminooxyacetate upon nutrient-stimulated insulin secretion. *Endocrinology* 111:392-397.

Malaisse WJ, Sener A, Welsh M, Malaisse-Lagae F, Hellerström C and Christophe J (1983) Mechanism of 3-phenylpyruvate-induced insulin-release. Metabolic aspects. *Biochem J* 210:921-927.

Matschinsky FM, Fertel R, Kotler-Brajtburg J, Stillings S, Ellerman J, Raybaud F and Holowach-Thurston J (1973) Factors governing the action of small calorigenic molecules on the islets of Langerhans, in *Proceedings of the 8th Midwest Conference on Endocrinology and Metabolism* (Breitenbach RP and Mussachia XJ eds) pp 63-87, University of Missouri, Columbia, MO.

Meyer M, Chudziak F, Schwanstecher C, Schwanstecher M and Panten U (1999) Structural requirements of sulphonylureas and analogues for interaction with sulphonylurea receptor subtypes. *Br J Pharmacol* 128:27-34.

Panten U, von Kriegstein E, Poser W, Schönborn J and Hasselblatt A (1972) Effects of L-leucine and α-ketoisocaproic acid upon insulin secretion and metabolism of isolated pancreatic islets. *FEBS* Lett :20:225-228.

Panten U, Schwanstecher M, Wallasch A and Lenzen S (1988) Glucose both inhibits and stimulates insulin secretion from isolated pancreatic islets exposed to maximally effective concentrations of sulfonylureas. *Naunyn-Schmiedeberg`s Arch Pharmacol* 338:459-462.

Panten U, Burgfeld J, Goerke F, Rennicke M, Schwanstecher M, Wallasch A, Zünkler BJ and Lenzen S (1989) Control of insulin secretion by sulfonylureas, meglitinide and diazoxide

in relation to their binding to the sulfonylurea receptor in pancreatic islets. *Biochem Pharmacol* 38:1217-1229.

Panten U, Heipel C, Rosenberger F, Scheffer K, Zünkler BJ and Schwanstecher C (1990) Tolbutamide-sensitivity of the adenosine 5´-triphosphate-dependent K^{+} channel in mouse pancreatic B-cells. *Naunyn-Schmiedeberg`s Arch Pharmacol* 342:566-574.

Sakmann B and Neher E (1983) Geometric parameters of pipettes and membrane patches, in *Single channel recording* (Sakmann B and Neher E eds) pp 37-51, Plenum Press, New York.

Schauder P, Herbertz L and Langenbeck U (1985) Serum branched chain amino and keto acid response to fasting in humans. *Metabolism* 34:58-61.

Schmeling H (2004) Direkte Hemmung ATP-sensitiver Kaliumkanäle der pankreatischen B-Zelle durch Monocarbonsäuren. Thesis, Technische Universität Braunschweig, Braunschweig, Germany; http://opus.tu-bs.de/opus/volltexte/2004/547.

Schwanstecher M, Schwanstecher C, Dickel C, Chudziak F, Moshiri A and Panten U (1994) Location of the sulphonylurea receptor at the cytoplasmic face of the β-cell membrane. *Br J Pharmacol* 113:903-911.

Sener A and Malaisse WJ (1980) L-leucine and a nonmetabolized analogue activate pancreatic islet glutamate dehydrogenase. *Nature* 288:187-189.

Tucker SJ, Gribble FM, Zhao C, Trapp S and Ashcroft FM (1997) Truncation of Kir6.2 produces ATP-sensitive K^{+} channels in the absence of the sulphonylurea receptor. *Nature* 387:179-183.

Zünkler BJ, Kühne S, Rustenbeck I and Ott T (2000) Mechanism of terfenadine block of ATP-sensitive K^{+} channels. *Br J Pharmacol* 130:1571-1574.

Footnotes

a)

This work was supported by the Deutsche Forschungsgemeinschaft.

b)

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

Mol 15388

Legends for figures

Fig. 1. Effects of α-ketoisocaproate and ATP on $K_{IR}6.2\Delta C26$ channel activity in an inside-out patch of a transfected COS-7 cell. The upper current traces in A and B show recordings of $K_{IR}6.2\Delta C26$ channel activity from the same membrane patch. Note lower channel activity in B compared to A due to channel run-down. 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 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 $K_{IR}6.2\Delta C26$ channel activity. Values for $N \cdot P_O$ were: 0.190 (control before α-ketoisocaproate application); 0.281 (15 mM α-ketoisocaproate); 0.331 (control after α-ketoisocaproate application). B, 0.3 mM ATP decreased $K_{IR}6.2\Delta C26$ channel activity by 57 %. Values for $N \cdot P_O$ were: 0.063 (control before ATP application); 0.049 (0.3 mM ATP); 0.164 (control after ATP application).

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 [3H]ligand binding were measured. For binding to recombinant SUR1 (filled symbols) the [3H]ligand was [3H]glibenclamide (0.3 nM), for binding to recombinant SUR2B (open symbols) the [3H]ligand was [3H]P1075 (3 nM). Results are expressed as percentage of control (absence of displacing drug). Symbols are means (with S.E. shown when larger than symbols) from 4-5 separate binding experiments. Analysis revealed the indicated IC_{50} values and Hill coefficients.

Mol 15388

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 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 caproate 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 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 Experimental procedures). Symbols are means (with S.E. shown when larger than symbols) from 4-10 experiments. Analysis revealed the indicated IC_{50} values and Hill coefficients. Data for α-ketocaproate and α-ketoisocaproate are taken from the thesis of Schmeling (2004) and have been obtained by the same method as applied in the present study.

29

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 8-10 (A) or 6-7 (B) separate experiments. From zero time to min 60 the islets were perifused with basal medium. A, from min 61 to min 104 the islets were perifused with basal medium containing 10 mM concentrations of β-phenylpyruvate (■), α-ketocaproate (△), α-ketoisocaproate (▲), or α-ketovalerate (○). B, from min 61 to min 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 min 60 the islets were perifused with basal medium containing 2.7 μM glipizide. From min 61 to min 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 min 60 the islets were perifused with basal medium containing 2.7 μM glipizide and 5 mM aminooxyacetate. From min 61 to min 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 (●).

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 1 molecule of α-keto acid anion: 1 NADH + 1 $FADH_2$ + 1 acetoacetate + 1 acetyl-CoA (consumption of 1 ATP) from α-ketoisocaproate; 2 NADH + 1 $FADH_2$ + 1 propionyl-CoA + 1 acetyl-CoA from α-ketocaproate; 2 NADH + 1 $FADH_2$ + 2 acetyl-CoA from α-ketovalerate; 2 NADH + 1 $FADH_2$ + 1 propionyl-CoA + 1 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. Since 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 and oxaloacetate 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 initiation of insulin secretion by stimulation of ATP production, the metabolism of α-keto acid anions amplifies the initiated insulin secretion.

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 4-6 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 (mM)	Hill	K_d (mM)	Hill	K_d (mM)	Hill
α-Ketoisovalerate	NM	NM	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

NM, not measured.

Figure 1

Figure 2

Figure 3

A

1mM ADP

1 mM ATP   20 mM PP   1 mM ATP
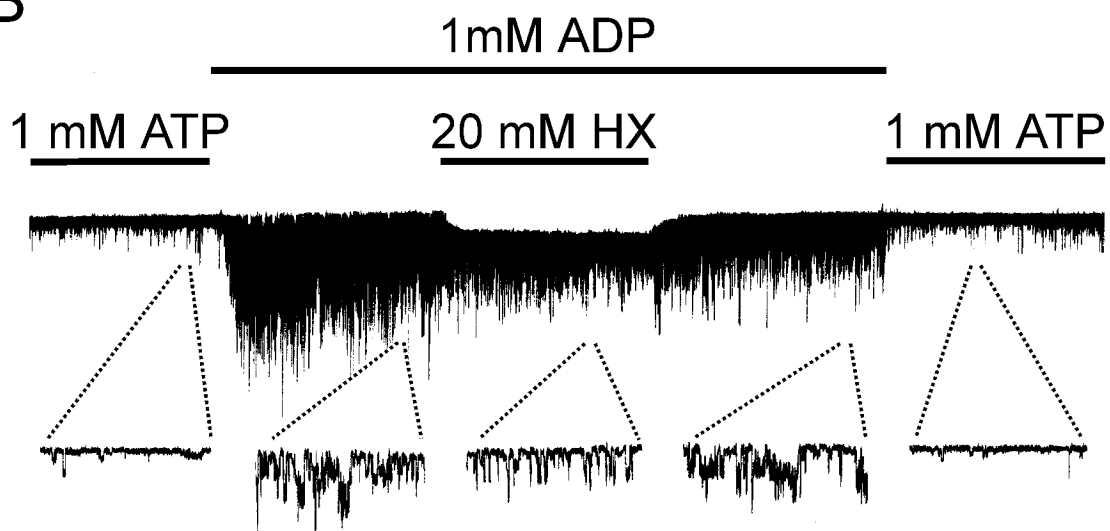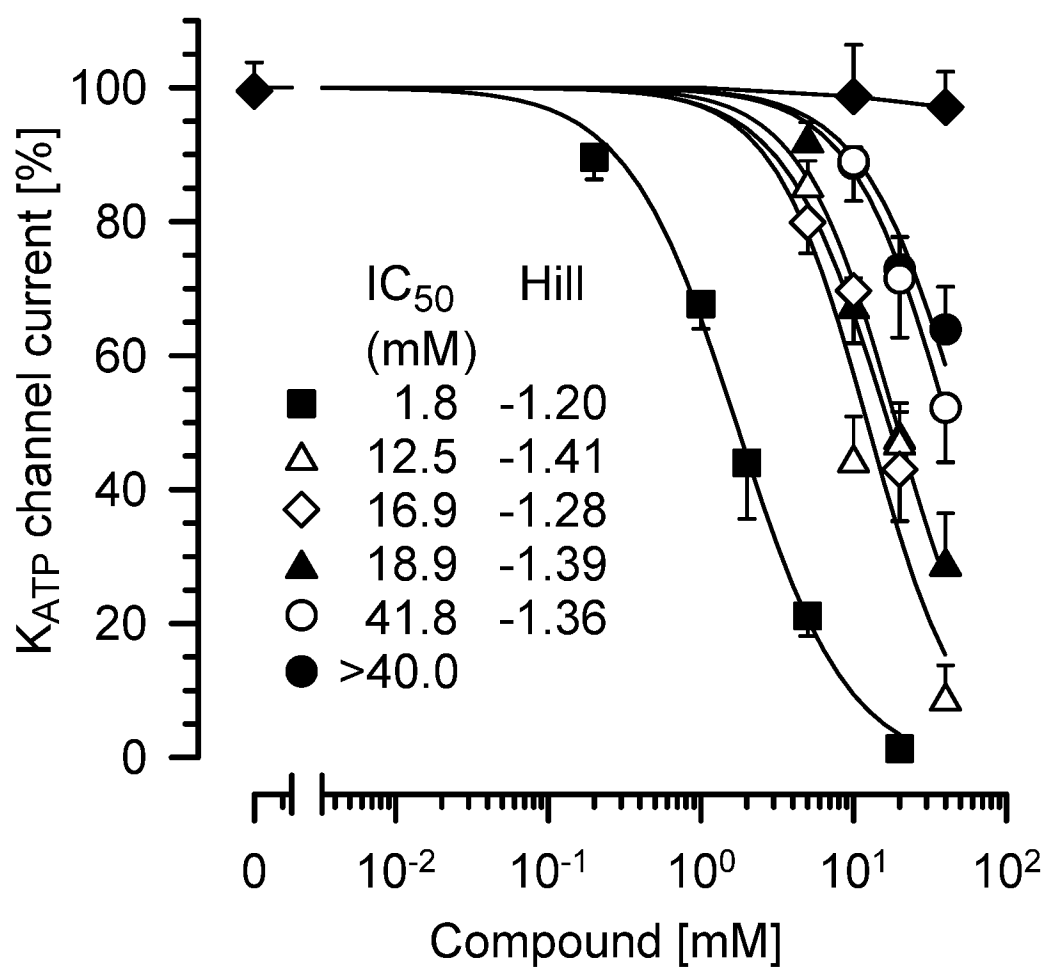
10 pA

B

1mM ADP

1 mM ATP   20 mM HX   1 mM ATP
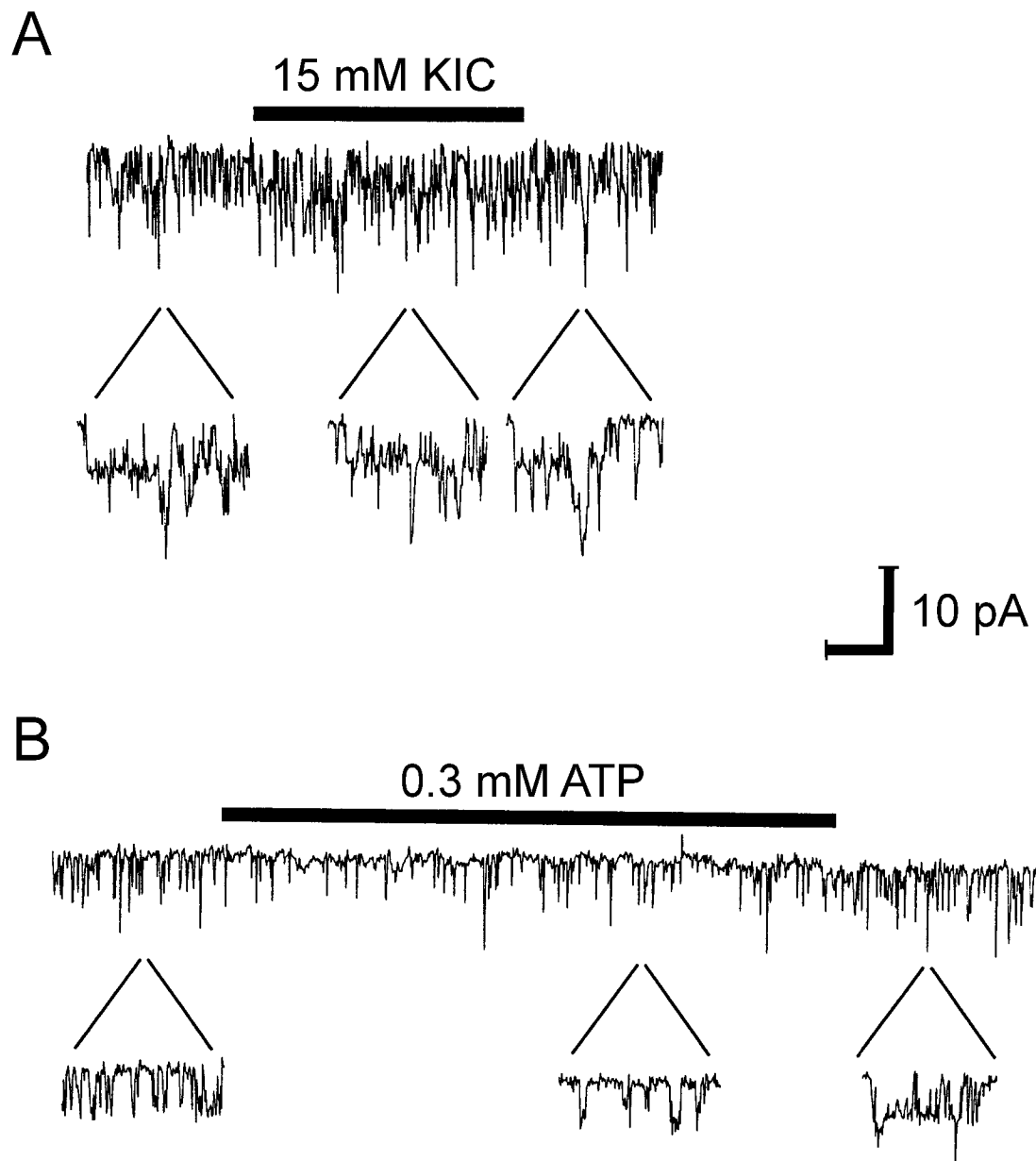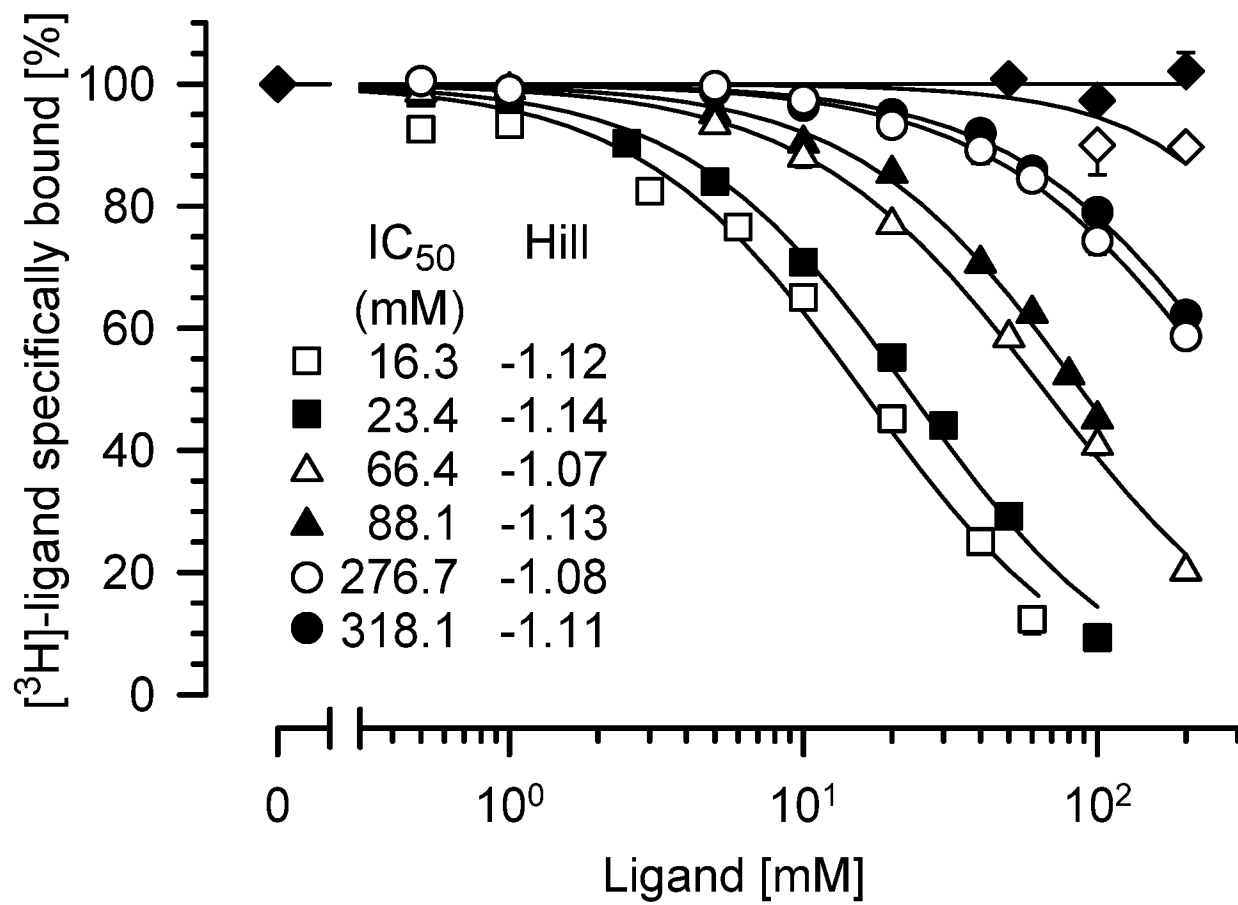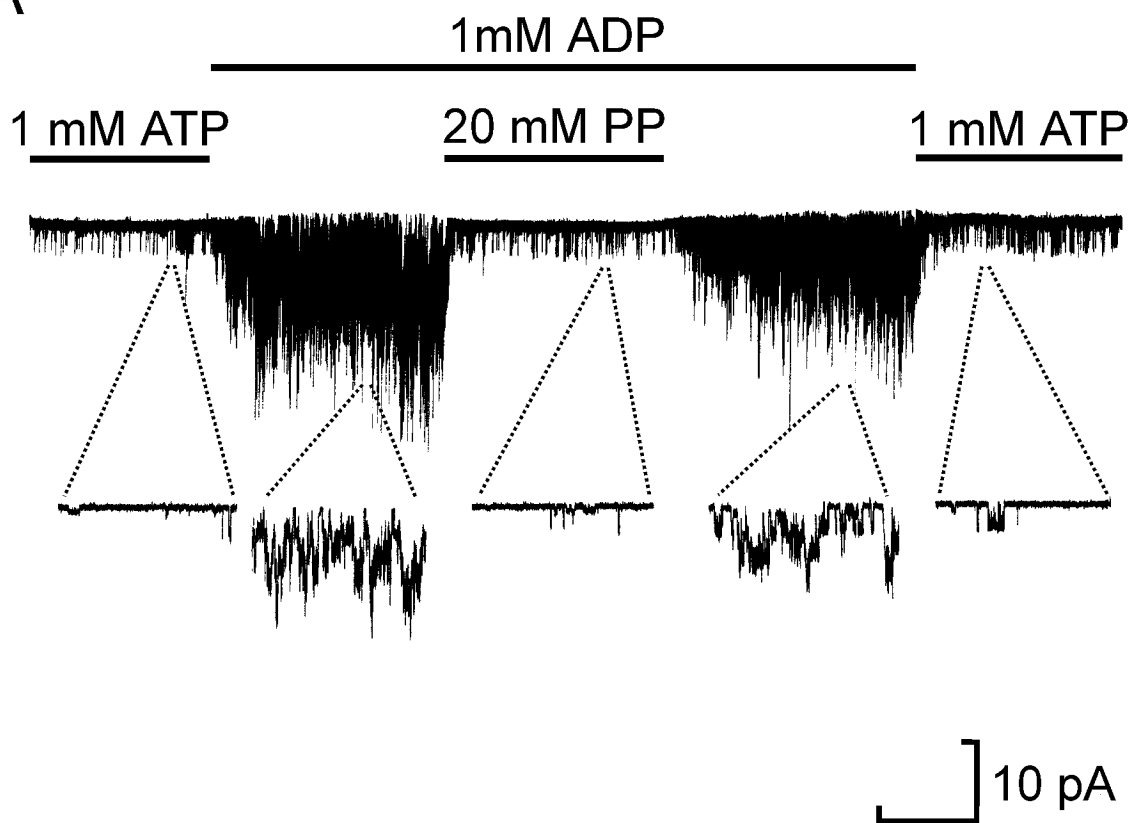
Figure 4

Figure 5

Figure 6

Figure 7