Biochemical Pharmacology 80 (2010) 104–112

Contents lists available at ScienceDirect

Biochemical Pharmacology

journal homepage: www.elsevier.com/locate/biochempharm

The signalling role of action potential depolarization in insulin secretion Metabolism-dependent dissociation between action potential increase and secretion increase by TEA

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ARTICLE INFO

Article history:
Received 14 January 2010
Accepted 11 March 2010

Keywords:
Cytosolic calcium concentration
Insulin secretion
Kv channel
K_{ATP} channel
Pancreatic islets
Plasma membrane potential

ABSTRACT

The K^+ channel blocker, TEA is known to increase action potential amplitude and insulin secretion of mouse β-cells when added to a nutrient secretagogue. In the presence of a maximally effective sulfonylurea concentration (2.7 μM glipizide) the nutrient secretagogue α-ketoisocaproic acid (KIC, 10 mM) strongly increased insulin secretion (about elevenfold). Instead of enhancing the effect of KIC, TEA reduced the KIC-induced secretion by more than 50%. Also, the secretion rate produced by 2.7 μM glipizide alone was significantly reduced by TEA. In contrast, TEA enhanced the insulinotropic effect of glipizide when a basal glucose concentration (5 mM) was present. In the presence as well as in the absence of glucose glipizide produced a plateau depolarization with superimposed action potentials. Under both conditions, TEA increased the glipizide-induced action potential amplitude and further elevated the cytosolic free calcium concentration ($[Ca^{2+}]_i$) with an oscillatory characteristic. These effects depended on the activity of L-type Ca^{2+} channels, even though the effect of TEA differed from that of 1 μM of the Ca^{2+} channel opener, Bay K8644, which primarily increased action potential duration. TEA did not negatively affect parameters of β-cell energy metabolism (NAD(P)H fluorescence and ATP/ADP ratio), rather, it slightly increased NAD(P)H fluorescence. Apparently, TEA inhibits insulin secretion in the absence of glucose in spite of a persistent ability to block K^+ ion conductance. Thus, the signalling role of action potential depolarization in insulin secretion may require reconsideration and ion conductance-independent actions of K^+ channels may be involved in this paradox effect of TEA.

1. Introduction

The stimulation of insulin secretion by nutrient secretagogues is accompanied by an electrical activity of the β-cell, which typically consists in a series of slow wave depolarizations with superimposed action potential spikes [1,2]. The slow wave depolarization was soon recognized to be due to a decrease in K^+ conductance [3]. The missing link between the nutrient-induced electrical activity and the activation of β-cell energy metabolism, which was known to be necessary for a stimulated insulin secretion, proved to be the existence of an ATP-sensitive K^+ channel in the β-cell plasma membrane [4,5].

However, experimental evidence suggests that K_{ATP} channel-mediated signalling is not the only pathway leading from increased energy metabolism (defined as the sequence from glycolysis via Krebs cycle to ATP synthesis) to stimulated insulin secretion. It could be shown that glucose caused a further stimulation of insulin secretion when K_{ATP} channels were blocked by sulfonylureas at maximally effective concentrations [6] or when β-cells were depolarized by a high K^+ concentration concomitantly with a permanent opening of the K_{ATP} channels by diazoxide [7,8]. Most likely, the export of citric acid cycle intermediates is involved in this additional pathway [9], but neither could a generally convincing signalling compound be identified nor has a final common target emerged. Also, the mechanisms of metabolic amplification may differ depending on the nutrient secretagogue: the prolonged exposure to a maximally effective concentration of the sulfonylurea, glipizide, in the absence of a nutrient abolished the response to a subsequent stimulation by 30 mM glucose, whereas 10 mM of the nutrient secretagogue α-ketoisocaproic acid (KIC) was still able to elicit a very strong amplifying effect [10,11].

Abbreviations: $[Ca^{2+}]_i$, cytosolic free calcium concentration; Kv channel, voltage-dependent K^+ channel; K_{ATP} channel, ATP-sensitive K^+ channel; KIC, α-ketoisocaproic acid; TEA, tetraethylammonium.

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doi:10.1016/j.bcp.2010.03.014

The additional pathway was originally named K_{ATP} channel-independent signalling pathway. Later the term "amplifying pathway" was proposed [12], because under physiological conditions it is unable to increase insulin secretion if the K_{ATP} channels remain open and thus is not truly independent on K_{ATP} channel function. The K_{ATP} channel-dependent pathway was proposed to be specifically responsible to produce the decisive triggering signal for exocytosis, namely the depolarization-induced Ca^{2+} influx [12], which is the correlate of the action potentials [13]. It is thus assumed that the immediate triggering signal corresponds to the amplitude between the β-cell resting membrane potential (between −70 and −60 mV) and the peak value of the action potentials which is in the range of −20 to +10 mV. This has led to the widespread use of experimental protocols where the triggering signal is imitated by imposed depolarizations of comparable magnitude, either by increasing the extracellular KCl concentration to 30, 40 or even 60 mM [14–16] or, in electrophysiological work, by repeated short-term current injection to depolarize from −70 to 0 mV [17].

An unexpected observation prompted us to reinvestigate the role of action potential depolarization in insulin secretion. To characterize the dependence of the metabolic amplification by the nutrient secretagogue KIC on the plasma membrane depolarization, 10 mM of the unspecific K^+ channel blocker TEA was added to a maximally effective concentration of the K_{ATP} channel blocker, glipizide. Since it is known that TEA increases the action potential amplitude, a more or less pronounced increase in secretion was expected. Surprisingly, TEA decreased the insulin release by KIC. It also decreased the glipizide-stimulated secretion when glucose was absent, but still elicited the typical effects on electrical activity. This discrepancy, which was confirmed using closely similar experimental conditions, suggests that action potential depolarization does not have a direct signalling role in insulin secretion. Since imposed depolarization is a common experimental tool to elicit insulin secretion the question as to which depolarization strength corresponds to physiological signalling is of major importance for the validity of the results.

2. Materials and methods

2.1. Chemicals.

TEA was obtained from Fluka (Buchs, Switzerland), glipizide and Bay K8644 from Sigma (Taufkirchen, Germany), tolbutamide from Serva (Heidelberg, Germany), Fura-PE3/AM from TEF Labs (Austin, TX, USA). Collagenase NB8 was purchased from Nordmark (Uetersen, Germany), cell culture medium RPMI 1640 from Gibco/Invitrogen (Karlsruhe, Germany) and fetal calf serum from PAA (Cölbe, Germany). ATP was determined by use of a luciferase luminescence kit (Sigma). All other reagents of analytical grade were from E. Merck (Darmstadt, Germany).

2.2. Tissue and cell culture

Islets were isolated from the pancreas of NMRI mice by a collagenase digestion technique and hand-picked under a stereomicroscope. Single cells were obtained by incubation of the islets for 10 min in a Ca^{2+}-free medium and subsequent vortex-mixing for 1 min. Islets and single islet cells were cultured in cell culture medium RPMI-1640 with 10% fetal calf serum (10 mM glucose until attachment, thereafter 5 mM glucose) in a humidified atmosphere of 95% air and 5% CO_2 at 37 °C.

2.3. Measurement of insulin secretion

Batches of 50 NMRI mouse islets were introduced into a purpose-made perifusion chamber (37 °C) and perifused with a HEPES-buffered Krebs-Ringer medium (2 mg/ml BSA) saturated with 95% O_2 and 5% CO_2, which contained the respective secretagogue. The insulin content in the fractionated effluate was determined by ELISA (Mercodia, Uppsala, Sweden).

2.4. Electrophysiological recordings.

The membrane potential of single β-cells was measured by a standard patch-clamp technique [18] using the perforated patch-configuration [19]. Pipettes were pulled from borosilicate glass (2 mm o.d., 1.4 mm i.d., Hilgenberg, Germany) by a two-stage vertical puller (List Electronic, Darmstadt, Germany) and had resistances between 3 and 6 MΩ when filled with solution. The compositions of the bath and pipette media were as given by Zünkler et al. [20]. Currents and voltages were recorded by an EPC 7 patch-clamp amplifier (HEKA, Lambrecht, Germany), and low pass-filtered by a 4-pole Bessel filter at 2 kHz and stored on a video tape. Exposure to the test compounds and wash-out was done by changing the bath medium with a slow bath perfusion system, mimicking the conditions of the secretion measurements. Thus, glucose in the bath (extracellular) medium was absent for at least 40 min before glipizide or TEA were added. Experiments were normally performed at room temperature (20–22 °C), selected experiments were repeated at 30–32 °C. Data were analysed off-line using pClamp 6.03 software (Axon Instruments, Foster City, CA, USA). The duration of the action potentials was measured at the half-maximal amplitude.

2.5. Microfluorimetric measurements of the cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$).

Intact islets from NMRI mice were cultured on collagen-coated glass cover slips in Petri dishes and were used from days 2 to 4 after isolation. Fura-PE3/AM was loaded at a concentration of 2 μM for 45 min at 37 °C. The cover slip with the attached islet was inserted in a purpose-made perifusion chamber (35 °C) on the stage of an epifluorescence microscope equipped with a thermostated objective (Zeiss Fluar 40x, 1.3 N.A.). The islet was perifused with a HEPES-buffered Krebs-Ringer medium, which was saturated with 95% O_2 and 5% CO_2 The fluorescence (excitation at 340 or 380 nm, emission >470 nm) was recorded by a slow-scan CCD camera (TILL Photonics, Gräfelfing, Germany).

2.6. Microfluorimetric measurements of NAD(P)H autofluorescence

The cell culture conditions of the NMRI islets, perifusion chamber, perifusion medium and the thermostated microscope objective were as for the $[Ca^{2+}]_i$ measurements. The NAD(P)H fluorescence (excitation by a Hanau St41 Hg arc lamp at 366 ± 15 nm, emission separated by a dichroic mirror at 405 nm and filtered by a 450 ± 32 nm bandpass) was registered by a photon-counting multiplier (Hamamatsu, Herrsching, Germany) at 1 Hz with 0.1 s exposure time.

2.7. Islet content of adenine nucleotides

15 freshly isolated islets were statically incubated at 37 °C to mimic perifusion conditions. Thereafter, proteins were precipitated and the adenine nucleotides extracted as described [11]. ATP was determined by use of the luciferase method. The ADP content of the extract was converted into ATP by the pyruvate kinase reaction, the difference between both measurements yielding the net ADP content.

2.8. Data handling and statistics

Statistical calculations were performed by Prism and Instat software (GraphPad, San Diego CA, USA). If not specified otherwise,

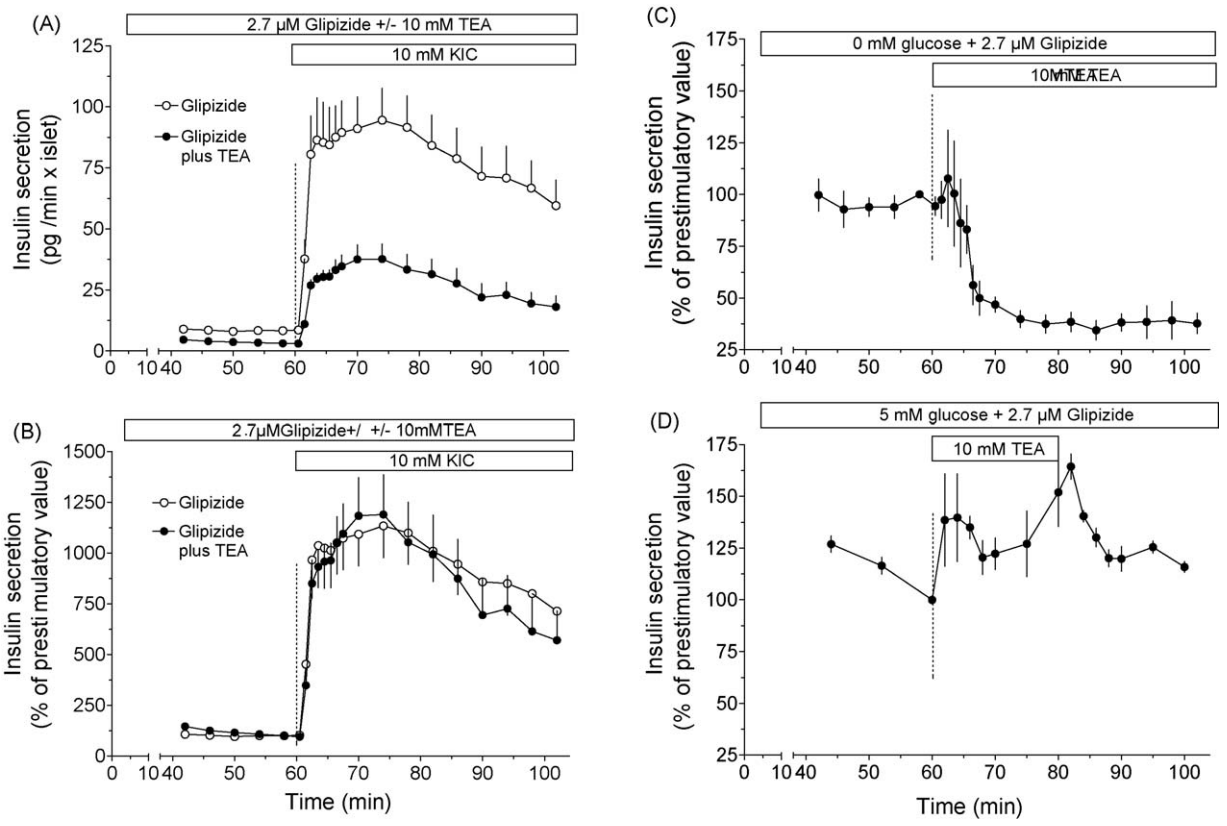

Fig. 1. Effect of 10 mM TEA on insulin secretion when added to a maximally effective concentration of glipizide. (A) Freshly isolated islets were perifused with Krebs-Ringer medium containing 0 mM glucose and 2.7 µM glipizide throughout (open circles) or, additionally, 10 mM TEA (closed circles). From 60 to 104 min 10 mM KIC was additionally present. Values are means ± SEM of six experiments. (B) Same experiments as in (A), the secretion data were normalized with respect to the last prestimulatory value (58 min). Note the unchanged relative increase of secretion by KIC. (C) Freshly isolated islets were perifused with Krebs-Ringer medium containing 0 mM glucose and 2.7 µM glipizide throughout. From 60 to 104 min the medium contained additionally 10 mM TEA. The secretion rate was reduced to about 40% within 12 min. Values are means ± SEM of 5 experiments. (D) Freshly isolated islets were perifused with Krebs-Ringer medium containing 5 mM glucose and 2.7 µM glipizide throughout. From 60 to 80 min the medium contained additionally 10 mM TEA. Values are means ± SEM of three experiments.

differences were considered significant if $p < 0.05$ (unpaired two-sided *t*-test).

3. Results

3.1. Inhibitory effect of TEA on insulin secretion

To check whether the insulinotropic effect of KIC in the presence of a maximally effective glipizide concentration (2.7 µM) can be enhanced by a further depolarization, 10 mM TEA was added to the sulfonylurea. Surprisingly, the strong amplifying effect of KIC (at the peak, 1130% of the rate of glipizide alone) was reduced by more than 50% under this condition (Fig. 1A). However, TEA also reduced the secretion rate prior to KIC exposure, so the relative increase of secretion by KIC remained unchanged (Fig. 1B). Thus, the effect of 10 mM TEA on the insulinotropic effect of 2.7 µM glipizide in the absence of glucose was tested. TEA reduced the glipizide-stimulated insulin secretion to a steady state of about 38% within 14 min (Fig. 1C). The onset of the inhibitory effect had a lag time of about 3–4 min. In the presence of 5 mM glucose, TEA had no inhibitory effect, rather, the glipizide-induced secretion was further enhanced with fast and reversible kinetics (Fig. 1D).

3.2. Effect of TEA on β-cell action potentials

TEA is known to increase the action potential amplitude elicited by physiological or pharmacological K_{ATP} channel blockers. A significant increase in action potential amplitude (from 21.2 ± 3.1 to 30.7 ± 3.7 mV, $n = 8$) by 10 mM TEA was observable in the presence of 2.7 µM glipizide and 0 mM glucose (Fig. 2A and B), suggesting that the ability of TEA to block K^+ channels was preserved under this condition. The addition of 10 mM KIC to the perifusion medium led to nearly complete repolarization within 1 min and a spontaneous recovery of depolarization with action potential spiking after 3–5 min (Fig. 2A). The spike amplitude and duration were not affected by the presence of KIC, except for the occasional occurrence of a very broad potential (>1 s duration) which was followed by a transient cessation of spiking activity (Fig. 2A, lower trace). The KIC-induced repolarization was also observed under control condition (only glipizide but no TEA contained in the medium, data not shown).

The increase in action potential amplitude by TEA was of the same magnitude (about 50% increase), when the medium contained 5 mM glucose and/or another sulfonylurea (tolbutamide, Fig. 2C). Of note, the increase in action potential duration (from 64.6 ± 6.2 to 103.3 ± 14.3 ms, $n = 4$) occurred only 2 or 3 min after the increase of the amplitude had begun. This increase was significant when the duration in the presence of TEA was referred to the duration in the absence for each single experiment ($p = 0.037$, unpaired *t*-test). There was no increase of the sulfonylurea-induced plateau depolarization by TEA, rather, action potentials with an increased amplitude were regularly followed by more marked repolarization (Fig. 2C, lower trace). Increasing the tolbutamide concentration from 50 to 500 µM significantly increased action potential frequency, but did not significantly affect the action potential amplitude (Fig. 2C). The increase in action potential amplitude by TEA was observed at 0 and at 5 mM glucose both at room temperature (20–22 °C) and at 30–32 °C, a temperature which permits insulin secretion.

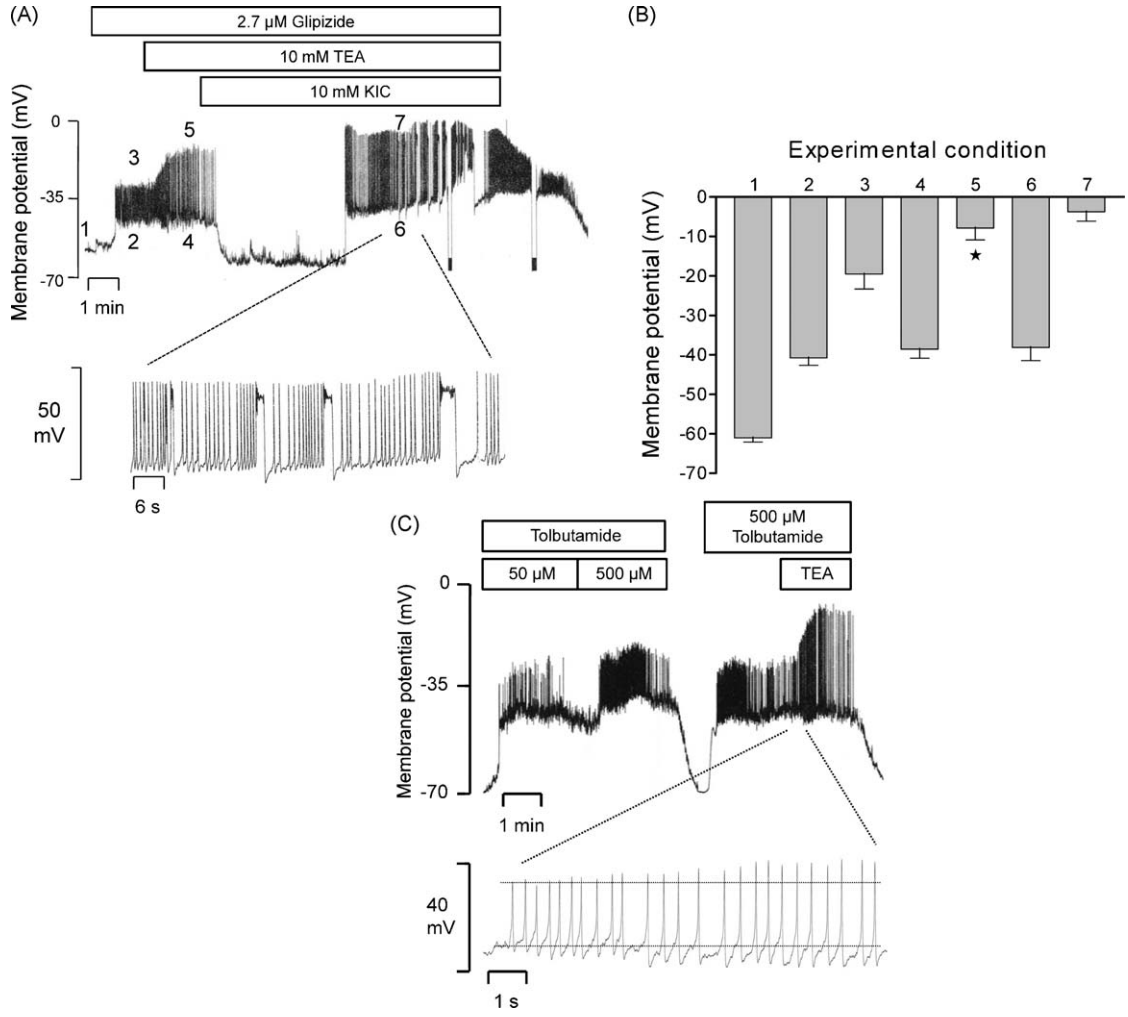

Fig. 2. Effect of TEA on the amplitude of action potentials induced by pharmacological and nutrient block of K_{ATP} channels. The membrane potential of primary pancreatic β-cells was measured in the perforated patch mode. Before start of the registration all cells had spent at least 40 min in the absence of glucose. (A) In the absence of glucose 2.7 μM glipizide produced a plateau depolarization with superimposed action potentials. 10 mM TEA increased the action potential amplitude by ca. 50%. The addition of 10 mM KIC led to a complete repolarization for about 3 min, thereafter, the depolarization reappeared. Note the occasional occurrence of prolonged action potentials which are followed by an undershooting repolarization and a decrease in action potential frequency (lower trace). (B) Mean values ± SEM of 8 experiments for the conditions as indicated by the numbers in (A). The asterisk denotes the significant increase in action potential amplitude by TEA. (C) In the presence of 5 mM glucose 50 μM tolbutamide produced a plateau depolarization with superimposed action potentials. Increasing the concentration to 500 μM caused a moderate increase in plateau depolarization and action potentials became more frequent but did not increase in amplitude. In contrast, when 10 mM TEA was added to 500 μM tolbutamide the amplitude increased by about 50%, the same effect as in the absence of glucose. Representative trace of five experiments.

3.3. Effect of TEA on β-cell membrane currents

The K_{ATP} channel blocking effect of glipizide in the absence of glucose was verified by measuring the current in response to hyper- and depolarizing steps of 10 mV [21]. At a holding potential of −70 mV 2.7 μM glipizide reduced membrane current strongly (by 83.4 ± 2.5%), but not completely (Fig. 3A), whereas 10 mM TEA had a only a slight inhibitory effect (by 15.4 ± 3.7%). Also, 10 mM TEA was unable to significantly depolarize the plasma membrane in the absence of a sulfonylurea (data not shown). When TEA was applied together with 2.7 μM glipizide and the holding potential was set at −50 mV no further reduction of the residual current was visible, rather, a marked inward current developed in response to the depolarizing steps (Fig. 3B). This current, which was observed both in the presence and absence of 5 mM glucose in the extracellular medium was abolished by 10 μM nifedipine which also abolished action potentials in intermittent current clamp registrations (Fig. 3B). It can thus be regarded to represent an L-type Ca^{2+} channel current.

3.4. Comparison with the effect of Bay K8644

The dihydropyridine opener of L-type Ca^{2+} channels, Bay K8644 (1 μM) produced a clearly different pattern of electrical activity than TEA. The action potential duration was significantly increased (from 70.7 ± 7.1 to 425.8 ± 51.1 ms), but the depolarization amplitude was not enhanced, rather, the peak value was even lower than in the absence of Bay K8644. The frequency decreased concomitantly with a more marked repolarization occurring after each action potential, which resulted in a lowering of the plateau depolarization by ca. 10 mV (Fig. 4A). When added to 10 mM TEA, Bay K8644 drastically prolonged the action potential (>2 s), each one being followed by an undershooting repolarization which prevented electrical activity until the next deformed action potential developed (Fig. 4B). On closer inspection the initial part of these deformed action potential consists of a fast sequence of action potentials with a progressive loss of repolarization together with a decrease in action potential amplitude (Fig. 4B, lower trace), a feature also found in the deformed action

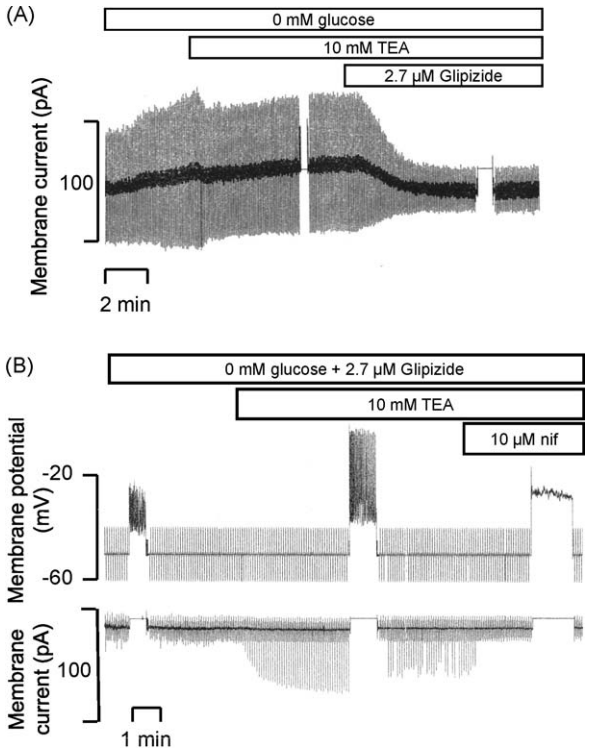

Fig. 3. Comparison of the effects of glipizide and TEA on whole cell currents and plasma membrane potential in perifused primary pancreatic β-cells. (A) Original recording of the effect of 10 mM TEA and, additionally, 2.7 μM glipizide on currents evoked by hyper- and depolarizing steps of 10 mV from a holding potential of −70 mV. Note the modest effect of TEA. Representative trace of 5 experiments. (B) Original recording of the effect of 10 μM nifedipine (nif) on currents evoked by hyper- and depolarizing steps of 10 mV from a holding potential of −50 mV in the presence of glipizide and TEA. The upper trace shows the membrane potential as controlled by the stimulation protocol, interrupted at three time points by current clamp measurements. Note the increase of action potential amplitude when the inward currents have developed by addition of TEA and the cessation of inward currents and action potential spiking within 1 min after addition of nifedipine (nif). Representative trace of four experiments.

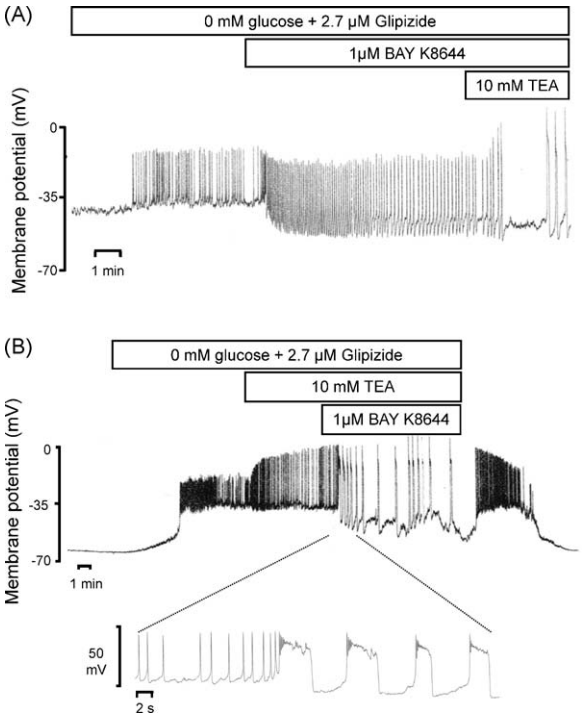

Fig. 4. Comparison of the effects of glipizide, TEA and Bay K8644 on action potentials in perifused primary pancreatic β-cells. (**a**) Original recording of the effect of 2.7 μM glipizide and, additionally, 1 μM of the Ca^{2+} channel opener, Bay K8644 on the membrane potential as registered in the perforated patch mode. Bay K8644 gradually increased action potential duration but not the peak depolarization. The undershooting repolarization was accompanied by a reduction of action potential frequency. Addition of 10 mM TEA increased the action potential amplitude and further increased the duration. Representative trace of four experiments. (B) Original recording of the effect of the combined effect of TEA and Bay K8644 on action potential amplitude and duration. While 10 mM TEA increased primarily the action potential amplitude, the additional presence of 1 μM Bay K8644 strongly increased action potential duration which was followed by an undershooting repolarization and a strong reduction of action potential frequency (lower trace). Representative trace of four experiments.

potentials produced occasionally by TEA in the presence of KIC (Fig. 2A).

3.5. Increase of $[Ca^{2+}]_i$ by TEA and Bay K8644 and the consequence for insulin secretion

In the absence of glucose 2.7 μM glipizide produced a sustained elevation of $[Ca^{2+}]_i$ and the addition of 10 mM TEA promptly induced an oscillatory pattern of $[Ca^{2+}]_i$ which persisted unchanged when glucose was raised to 5 mM (Fig. 5A). When the effect of glipizide and TEA was tested separately in the presence (5 mM) and absence of glucose, the mean values were superimposable (data not shown). In particular, no decrease in $[Ca^{2+}]_i$ could be noted in these experiments which would have explained the inhibitory effect of TEA on insulin secretion. The difference between TEA and Bay K8644 in their effect on electric activity was reflected by different $[Ca^{2+}]_i$ patterns. TEA induced a $[Ca^{2+}]_i$ oscillation on top of the $[Ca^{2+}]_i$ plateau established by glipizide, which resulted in a moderate increase of the mean value. The addition of 1 μM Bay K8644 led to a more pronounced increase of the mean $[Ca^{2+}]_i$ value, thereby reducing the oscillation and establishing a further elevated plateau of $[Ca^{2+}]_i$ (Fig. 5B). However, even the constantly elevated $[Ca^{2+}]_i$ level by TEA plus Bay K8644 did not affect the TEA-induced decrease of the secretion rate. As in the initial experiments (Fig. 1) TEA reduced the glipizide-stimulated secretion of perifused islets to less than 50% at which level it remained unchanged after addition of 1 μM Bay K8644 (Fig. 5C).

3.6. β-cell energy metabolism in the presence of TEA

Since insulin secretion is strongly dependent on an intact energy metabolism of the β-cell, a possible inhibitory effect of TEA was explored by measuring the islet content of ATP and ADP and the islet NAD(P)H autofluorescence. The measurement of the adenine nucleotides was performed on statically incubated islets where the conditions were chosen to fit to the secretion measurements in Fig. 1. Neither in the presence of 0 mM nor of 5 mM glucose did TEA decrease the adenine nucleotide content or the ATP/ADP ratio (Fig. 6A). The only significant change of the ratio was the increase produced by 5 mM glucose vs 0 mM glucose ($p < 0.01$, Wilcoxon signed rank test, Fig. 5A). The absence of glucose for 1 h had a comparatively moderate effect on the adenine nucleotide content as becomes clear by comparison with the effect of the uncoupler of oxidative phosphorylation, CCCP (Fig. 6B). Finally, TEA did not diminish the NAD(P)H increase caused by 10 mM KIC (Fig. 6A) after 1 h perifusion in the absence of glucose. Unexpectedly, glipizide itself led to an increase of the NAD(P)H autofluorescence of perifused islets, the amplitude of which was slightly increased by the additional presence of TEA (Fig. 7A). When tested alone, 10 mM TEA produced in fact a modest NAD(P)H fluorescence increase in the presence of 0 mM but not of 5 mM glucose (Fig. 7B).

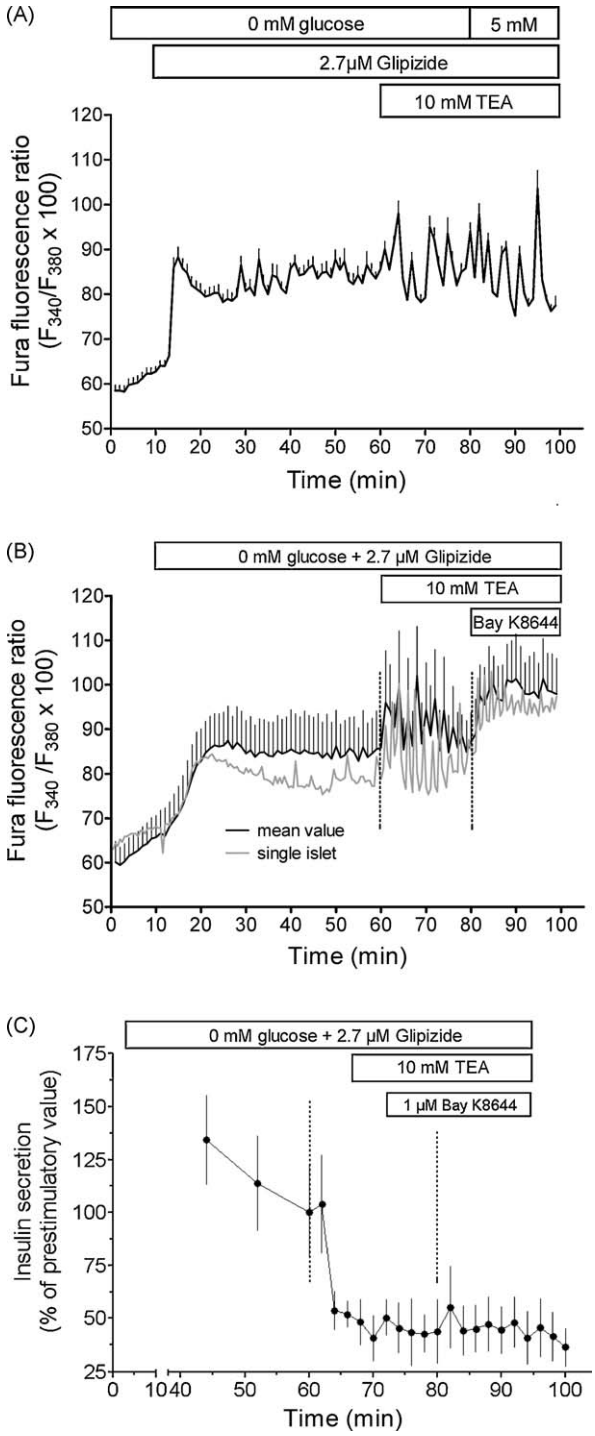

Fig. 5. Effect of glipizide, TEA and Bay K8644 on the cytosolic calcium concentration $[Ca^{2+}]_i$ and insulin secretion. (A) Single Fura-PE3-loaded isolated islets were perifused with Krebs-Ringer medium containing initially 0 mM glucose. From 10 min on the medium contained 2.7 μM glipizide and from 60 min on additionally 10 mM TEA. After 20 min of exposure to TEA glucose was raised to 5 mM. Values are means ± SEM of four experiments. (B) Comparison of the effect of TEA and of the Ca^{2+} channel opener, Bay K8644 at a higher time resolution. Single Fura-PE3-loaded isolated islets were perifused with Krebs-Ringer medium containing 0 mM glucose throughout. From 10 min on the medium contained 2.7 μM glipizide, to which 10 mM TEA was added from 60 min on and 1 μM Bay K8644 from 80 min on. Values are means ± SEM of four experiments (black trace), a single experiment (grey trace) is depicted to more clearly show the TEA-induced oscillation. (C) Freshly isolated islets were perifused with Krebs-Ringer medium containing 5 mM glucose and 2.7 μM glipizide throughout. From 60 min to 80 min the medium contained additionally 10 mM TEA and from 80 to 100 min 10 mM TEA plus 1 μM Bay K8644. Values are means ± SEM of three experiments.

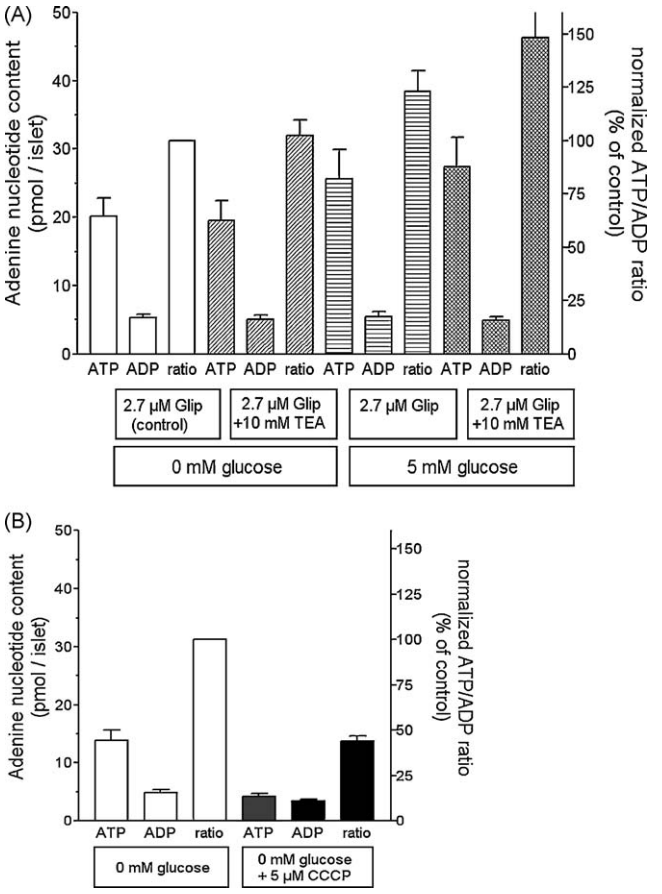

Fig. 6. ATP and ADP contents of statically incubated mouse islets. (A) As in the perifusion experiments, the medium contained either 0 or 5 mM glucose. In each case 2.7 μM glipizide (glip) was present. After an incubation of 60 min, TEA or Krebs-Ringer medium was added and the incubation was continued for another 15 min. Compared with the incubation in the absence of glucose, the presence of 5 mM glucose led to a significant increase of the normalized ATP/ADP ratio ($p = 0.008$ for glipizide alone or $p = 0.027$ for glipizide plus TEA, Wilcoxon signed rank test). TEA had no significant effect on the ATP/ADP ratio. Values are means ± SEM of eight experiments. (B) Compared with the prolonged absence of glucose (1 h) the uncoupler of oxidative phosphorylation, CCCP, affected adenine nucleotides much more strongly, reducing the ATP content to 30% and the ATP/ADP ratio to 44%. Such islets no longer respond to fuel stimuli. Values are means ± SEM of eight experiments.

4. Discussion

This study demonstrates a hitherto unknown inhibitory effect of TEA on insulin secretion. This effect becomes visible when two conditions are met: first the absence of glucose, second a decrease in K_{ATP} channel activity, enabling the electrical activity of the β-cells. Since we found the well-known effect of TEA on the electrical activity of the β-cell to be preserved under these conditions, there is a dissociation which puts the signalling role of action potential depolarization for insulin secretion into question.

It is generally accepted that the Ca^{2+} channel activity of the β-cell is reflected by the action potentials appearing on top of the slow wave depolarizations caused by K_{ATP} channel closure [2]. The duration of the action potentials is limited by the opening of voltage-dependent K^+ channels (Kv channels) and of Ca^{2+}-dependent K^+ channels (BK and SK channels), the latter apparently contributing less than the earlier (ca 20% vs 80% [22]). The well-established effect of TEA on the activity of electrically excitable cells is to block the ion conductance of voltage-dependent K^+ channels, which in β-cells finds its expression in an increased action potential amplitude and duration and a decreased

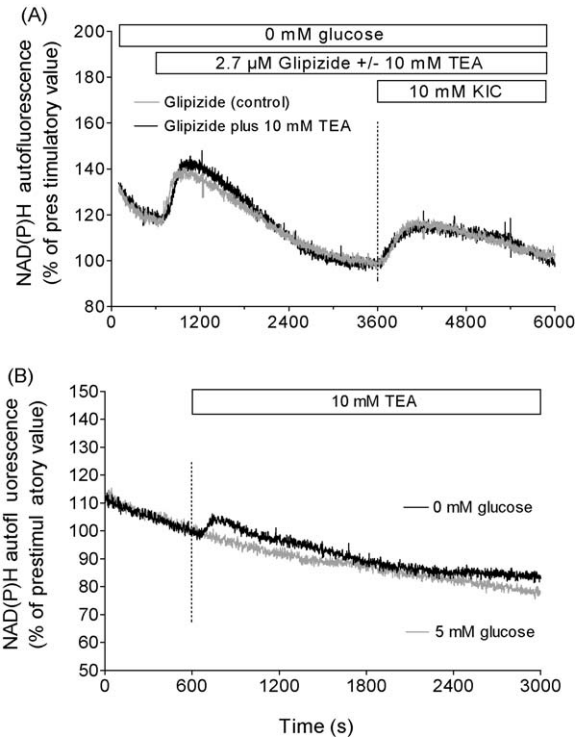

Fig. 7. Effect of glipizide and TEA on the NAD(P)H fluorescence of single perifused islets. (A) Single cultured islets were perifused with Krebs-Ringer medium without glucose. From 600 s on the medium contained 2.7 µM glipizide (grey trace) or, additionally 10 mM TEA (black trace). From 3600 s on 10 mM KIC was added as an insulin secretagogue. The presence of TEA did not cause a significant change of the KIC-induced increase of NAD(P)H fluorescence. Data are mean values of three perifusions each. (B) Single cultured islets were perifused with Krebs-Ringer medium containing no glucose (black trace) or 5 mM glucose (grey trace). From 600 s on the medium contained 10 mM TEA. Note the complete absence of TEA effect in the presence of 5 mM glucose. Data are mean values of three perifusions each.

frequency [23]. TEA is a fairly unspecific K^+ channel blocker, in principle Kv channels, BK channels and K_{ATP} channels can be inhibited [24,25]. However, the potency to block K_{ATP} channels is markedly lower than to block Kv channels [25]. At the concentration used in this study (10 mM) TEA only modestly reduced the current evoked by hyper- and depolarizing steps at the resting membrane potential and was practically unable to depolarize the β-cell membrane potential. In consequence, the effect on K_{ATP} channels can be assumed to be negligible under our conditions.

TEA has repeatedly been shown to enhance the insulinotropic effect of glucose [26,27]. Since the block of Kv channels increases action potential amplitude and duration, an increased Ca^{2+} influx via voltage-dependent Ca^{2+} channels and, consequently, an enhanced secretion appear as logical consequences. A glucose-dependent enhancing effect on insulin secretion was also observed with more specific blockers of Kv channels lending support to this hypothesis [28]. Concurrent with these earlier observations we found TEA to increase the insulinotropic effect of glipizide in the presence of a basal glucose concentration (5 mM). The paradoxical inhibitory effect in the absence of glucose has not yet been described. However, in an earlier investigation a lack of insulinotropic effect of 20 mM TEA was noted when a very high concentration of glibenclamide was used to block K_{ATP} channels in the presence of a low glucose concentration [27]. Since this loss of effect was not observed with an insulin-secreting cell line, it was concluded that unspecific effects of the high sulfonylurea concentration on non-β-cells in the islets were responsible [27].

From our present observations we would rather conclude that the low glucose concentration is the critical factor which may not only lead to a loss of the insulinotropic effect, but may even reveal an inhibitory effect of TEA. Of note, the prolonged (1 h) absence of glucose together with a constant sulfonylurea stimulus is an experimental condition which does not impede the secretory responsiveness as such [11]. This can be seen from the immediate and strong response to 10 mM KIC (Fig. 1) ruling out a depletion of the pool of readily releasable granules [29]. The sulfonylurea-induced secretion is known to be fuel-dependent [30], therefore only a moderately enhanced secretion resulted from the maximally effective concentration of glipizide prior to KIC exposure. Nevertheless, the preserved effect of glipizide on K^+ currents and the increase in $[Ca^{2+}]_i$ suggest that the insulinotropic effect of glipizide in the absence of glucose was due to the classical mechanism of action of the sulfonylureas.

Since the typical effect of TEA on action potentials was still visible after prolonged absence of glucose it could be expected that, as a consequence, an increased Ca^{2+} influx occurred. In fact, the $[Ca^{2+}]_i$ response pattern produced by sequential addition of glipizide and TEA was quite similar in the absence and presence of glucose. When expressed as the mean value of several experiments TEA caused a moderate further $[Ca^{2+}]_i$ increase in addition to that evoked by glipizide. In single islet recordings an oscillatory pattern was clearly visible, which fits to the earlier observations that TEA transformed slow $[Ca^{2+}]_i$ waves by 20 mM glucose into a fast oscillatory pattern [31] and produced a $[Ca^{2+}]_i$ oscillation on top of the elevated $[Ca^{2+}]_i$ plateau established by 1 mM tolbutamide [32]. Interestingly, the addition of Bay K8644 ceased the TEA-induced oscillation and established a further elevated plateau. The increase in action potential duration caused by Bay K8644 is apparently particularly effective to produce a sustained increase in $[Ca^{2+}]_i$ even though the prolongation of the action potential was linked to a drastic decrease in frequency. Thus, the relation between action potential duration and increase in $[Ca^{2+}]_i$ may be more complex than thought.

The inward current, which became visible when TEA was added to glipizide at a partially depolarized holding potential (−50 mV) could be identified as L-type Ca^{2+} channel activity (Fig. 3C) which is generally recognized as an indispensable signal transducer for the initiation of insulin secretion [12,33]. The concomitant increase in action potential amplitude seen in these experiments (Fig 3B) may thus be due to the depolarizing influx of Ca^{2+} which in the presence of TEA was no longer offset by a K^+ efflux via Kv or BK/SK channels. Thus, action potential amplitude is not a direct measure of the magnitude of Ca^{2+} influx. In fact, a strong Ca^{2+} influx via voltage-dependent Ca^{2+} channels can occur without any action potential firing, as is the case with K^+ depolarization. Under this condition the effect of Ca^{2+} influx on the membrane potential can be entirely offset by the accelerated K^+ efflux via open K_{ATP} channels [34]. Given these considerations neither the action potential amplitude nor the peak depolarization should be taken as an independent parameter regulating the triggering pathway of insulin secretion.

Since the effect of TEA on the membrane depolarization pattern and its direct consequences on the triggering pathway were found to be unaffected by the absence of glucose, we supposed that TEA might have an independent additional effect on β-cell metabolic signalling. It is known that an inhibition of mitochondrial energy metabolism can abolish both, nutrient- and depolarization-induced insulin secretion [35]. However, TEA did not decrease the adenine nucleotide content nor did it decrease the ATP/ADP ratio. Also, the assumption that the combination of TEA and glipizide would inhibit the KIC-induced production of NADH [36,37] proved to be incorrect.

Rather, both TEA and glipizide increased the NAD(P)H fluorescence by themselves. Since the increase by TEA, modest as it was, was only visible in the absence of glucose, it is unlikely to

represent an increased production of mitochondrial reducing equivalents. Given the complex nature of the cellular NAD(P)H autofluorescence [37] it may also result from changes in cytosolic NADPH levels which would be functionally relevant in view of the reported redox (NADPH)-dependence of Kv channels [22]. But why is secretion inhibited when the TEA effect on K^+ ion conductance and its consequence on $[Ca^{2+}]_i$ are obviously unchanged?

Apparently, there is some sort of uncoupling between Ca^{2+} influx via L-type Ca^{2+} channels and exocytosis, as can be seen from the use of the specific channel opener, Bay K8644. In spite of the clear increase of $[Ca^{2+}]_i$ beyond the levels established by K_{ATP} channel closure and by K_v channel block, no increase of the TEA-diminished secretion could be produced. In this regard, the recent reports are intriguing that Kv2.1 channels may have a facilitating role in insulin secretion which is independent of their ion-conducting function and which may involve interactions with the cytoskeleton and SNARE proteins [38,39]. In particular, N-terminal domains of the SNARE protein SNAP-25 have been described to interact with Kv2.1 channels and to affect the sensitivity to TEA block [40]. So it is conceivable that vice versa TEA might affect the function of SNAP-25, depending on the presence or absence of glucose. The idea that such a very distal step in insulin secretion is affected is supported by the observation that both, depolarization-induced secretion (glipizide in the absence of glucose) and depolarization-independent enhancement of secretion (amplifying effect of KIC) were similarly inhibited by TEA (Fig. 1B).

Summing up the above, the present observations suggest that action potential amplitude (or the peak depolarization value) is not an independent parameter determining insulin secretion, even if only triggering mechanisms are considered. An increase in action potential amplitude may occur when insulin secretion is actually inhibited (this paper) and vice versa Ca^{2+}-induced insulin secretion may occur without any action potential spiking [33]. Also, TEA appears to affect insulin secretion by other mechanisms than by inhibiting K^+ ion conductance which adds circumstantial evidence to the hypothesis that voltage dependent K^+ channels are regulators of the exocytotic machinery in β-cells.

Acknowledgments

This work was supported by grants from the Deutsche Forschungsgemeinschaft (Ru 368/5-1), the Deutsche Diabetes Gesellschaft and the Deutsche Diabetes Stiftung. Skilful technical assistance by Angela Hahlbohm, Verena Lier-Glaubitz and Sabine Warmbold is gratefully acknowledged.

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